FLOWERS, SEX, AND DIVERSITY. REPRODUCTIVE-ECOLOGICAL AND MACRO-EVOLUTIONARY ASPECTS OF FLORAL VARIATION IN THE PRIMROSE FAMILY, PRIMULACEAE

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CONTENTS

ZUSAMMENFASSUNG

Es ist ein zentrales Ziel in der Evolutionsbiologie, die Muster der Vielfalt und die Prozesse, die sie erzeugen, zu verstehen. Die enorme Vielfalt der Blütenpflanzen (wahrscheinlich> 352'000 Arten) zeigt sich auch in der Vielfalt der Blumen, Blütenstände und reproduktiven Strategien. Wichtige Theorien der Diversifizierung der Blütenpflanzen behaupten, dass Blütenmerkmale, die dazugehörigen Bestäuber-Syndrome und Sexualsysteme, die wichtigsten Triebkräfte der Diversifizierung der Blütenpflanzen darstellen. Insbesondere evolutionäre Übergänge zwischen Auskreuzung und Inzucht - einer der häufigsten Übergänge in der Blütenpflanzenevolution – haben der Theorie gemäss einen staken Einfluss auf das Muster der Anhäufung neuer Arten und Eigenschaften im Laufe der Zeit. Allerdings ist die Rolle der Blütenmerkmale in der Evolution der pflanzlichen Reproduktionsvielfalt nur teilweise verstanden.

In dieser Arbeit verband ich Methoden aus den Bereichen der reproduktiven Ökologie und molekularen Phylogenetik um die Evolution der pflanzlichen Reproduktionsvielfalt zu untersuchen. Ich erforschte die Sexualsysteme "Heterostylie" und "Homostylie" in der Familie der Schlüsselblume (Primulaceae). Heterostylie ist ein genetischer Polymorphismus, der dazu führt dass Populationen aus zwei (Distylie) oder drei (Tristylie) Blütenmorphen bestehen, die sich durch die Position der Antheren (Staubblätter) und Narbe (Stigma) in der Blüte unterscheiden. In der Regel führt nur eine Bestäubung zwischen den Blütenmorphen zu völlig fertilen Nachkommen. Dem entsprechend sind heterostyle Pflanzen abhängig von Pollenvektoren für eine erfolgreiche Fortpflanzung. In vielen der ca. 28 Familien mit Heterostylie kommen auch homostyle Arten vor, welche selbst-kompatibel sind und allgemein als höchst selbst fruchtbar gelten. Der Übergang von Heterostylie zu Homostylie ist beispielhaft für den Verlust von Selbst-Inkompatibilität und die Entwicklung der Selbstbefruchtung in Blütenpflanzen.

In Kapitel 2, teste ich die Hypothese, dass die Entwicklung der Heterostylie die Rate erhöht, mit der Arten im Laufe der Zeit in der Schlüsselblumefamilie angesammelt werden. In dieser Studie erstellte ich eine Phylogenie der Primulaceae, die die Diversität gut repräsentiert, da 265 Taxa verwendet wurden, was 36% der existierenden Artenvielfalt entspricht. Die Phylogenie stellt das bisher genauste Bild der evolutionären Dynamik und Diversifikation in Primulaceae dar. Ich zeigte einen stark abgestützten Zusammenhang zwischen der Entwicklung der Heterostylie und einer höhere Rate der Artendiversifizierung, und zeigte dass dies durch eine geringere Aussterberate erklärt wird, und nicht durch eine höhere Artbildungsrate. Außerdem fand ich, dass die evolutionären Gewinne und Verluste der Heterostylie sich unterschiedliche auswirken auf das Muster der Diversifikation, wenn man über kurze oder lange evolutionäre Zeiten schaut. Insgesamt können die Ergebnisse als Beweis für die langfristigen positiven Effekte der Auskreuzung interpretiert werden.

In Kapitel 3, habe ich Merkmalsdiversifizierung anstatt Artendiversifizierung angeschaut. Die Verschiebung von Auskreuzung Richtung erhöhte Selbstung ist typischerweise mit Änderungen in mehreren Blütenmerkmalen verbunden, dem sogenannte Selbstungssyndrom, einschließlich einer Verringerung der Blütengrösse. Ich habe kürzlich entwickelte vergleichende Methoden zur

quantitativen Auswertung des Übergangs von Heterostylie zu Homostylie für vier Blütenmerkmale und 126 Primelarten eingesetzt. Entgegen den Erwartungen, fand ich ähnliche Variabilität in Blüten mit und ohne Heterostylie, aber Unterschiede zwischen den Merkmalen: homostyle Blüten sind kleiner in einigen, aber nicht allen Dimensionen. Die Merkmalsevolution nach dem Verlust der Heterostylie erklärt sich am besten durch eine deutliche Steigerung in der Intensität der stochastischen Schwankungen in der Evolution - eine unerwartetes Ergebnis. Diese Ergebnisse lassen sich erklären durch eine erhöhte Bedeutung der Drift in der Evolution der Blütenmorphologie nach dem Verlust des Selbst-Inkompatibilität.

In Kapitel 4, habe ich mir die reproduktiven Auswirkungen der Variation der Blütenmorphologie im Alpenraum am Beispiel der homostylen Art *Primula halleri* angeschaut. Es wird behauptet, dass unzuverlässige Dienst der Bestäuber dazu führen, dass die Entstehung von selbst-kompatiblen Sexualsystemen wie Homostylie bevorzugt wird, weil Selbstung reproduktive Sicherheit bringt, wenn die Möglichkeiten der Auskreuzung begrenzt sind, z. B. im alpinen Lebensraum. Jedoch können männliche und weibliche Sexualorgane der homostylen Arten ebenfalls räumliche Trennung (Herkogamie) aufweisen, eine Anordnung welche Auskreuzung fördert. Umfangreiche Bestäubungsexperimente und morphologische Untersuchungen ergaben, dass die Herkogamie während der Blütezeit abnimmt, aber das absolute Ausmass der Herkogamie in reifen Blüten unterscheidet sich zwischen Individuen und Populationen. Experimente mit Bestäuberausschluss zeigten, dass Herkogamie das Potenzial für autonome Selbstung reduziert, und Vergleiche zwischen Emaskulationsbehandlunge und freier Bestäubung zeigten, dass erhöhte Herkogamie den totalen Samenproduktion und das Potenzial für reproduktive Sicherheit deutlich abnehmen lässt. Diese Studie legt nahe, dass auch kleine Unterschiede von Herkogamie große Auswirkungen auf den reproduktiven Erfolg homostyler Arten haben. Deswegen wäre es durchaus möglich das homostyle Arten mehr Auskreuzen als allgemein angenommen wird.

In Kapitel 5, untersuchte ich die evolutionären Beziehungen zwischen den sieben Arten *Primula* Sektion *Primula*, zu der namhafte europäische Arten, Primeli, Schlüsseli, gehören. Ich analysierte einen Datensatz Nukleärer- und Chloroplasten-DNS, der alle Arten und Unterarten enthielt und geografisch repräsentativ gesammelt war. Ich benutzte mehreren Bayesianische Methoden um Genbäume und Artenbäume zu rechnen. Insbesondere habe ich die Wirkung von ungenauer Spezifität der Prior auf die Berechnung der Branchlengths untersucht. Diese führte häufig zu artefaktischen Ergebnissen, die oft übersehen werden. Fehlspezifikation kann dazu führen das Bäumen bis zu zwei Größenordnungen zu lange waren, aber topologische Beziehungen waren davon nicht betroffen. Die Phylogenie zeigte extreme genetische Heterogenität innerhalb der Arten auf und auch hohe Non-monophyly der Arten, vor allem für *Primula elatior*. Die Muster in den Genbäume sind in Übereinstimmung mit der Interpretation, dass *P. elatior* in seiner aktuellen Umschreibung das unzusammenhängende Überbleibsel einer uralten Spezies ist, aus welcher andere anerkannte Arten entstanden sind.

Die Forschung in dieser Arbeit zeigt die Wirksamkeit eines integrierten evolutionären Vorgehens, weil sie, soweit angebracht, Verbindungen zwischen Ökologie und Makroevolution schafft. Ökologische Funktion von Merkmalen und phylogenetische Muster können so einander gegenseitig

bestätigen. Damit liefert diese Arbeit einen Beitrag zu dem übergeordneten Ziel der Aufklärung der Prozesse, welche in der Entwicklungsgeschichte der Primulaceae verantwortlich sind für ihre Diversifizierung, und zu dem Ziel wie wir die Entwicklung der pflanzlichen Reproduktionsvielfalt erklären können.

SYNOPSIS

It is a central goal in evolutionary biology to understand the patterns of diversity and the processes that generate it. The tremendous diversity of flowering plants (probably > 352,000 species) is especially evident in the variety of flowers, inflorescences and reproductive strategies. Floral innovations, their associated pollinator syndromes and plant breeding systems have been proposed as the main drivers of the diversification of flowering plants. In particular, transitions between cross-breeding and inbreeding - one of the most common transitions in flowering plant evolution - are hypothesized to strongly affect the pattern of accumulation of new species and traits over time. However, the role of floral traits in the evolution of plant reproductive diversity remains poorly understood.

In this thesis, I combine methodologies from the fields of reproductive ecology and molecular phylogenetics to contribute to the understanding of the evolution of plant reproductive diversity, by using the breeding systems "heterostyly" and "homostyly" in the primrose family (Primulaceae) as a study system. Heterostyly is a genetic polymorphism in which plant populations are composed of two (distyly) or three (tristyly) morphs that differ reciprocally in the position of anthers and stigmas in flowers, termed reciprocal herkogamy, which is usually associated with a self- and intra-morph incompatibility system. Thus, heterostylous plants depend on pollen vectors and mates for successful reproduction. In many of the ca. 28 families with heterostyly, homostylous species occur, which are self-compatible and commonly assumed to be highly self-fertile. The transition from heterostyly to homostyly is exemplary for the loss of self-incompatibility and evolution of selfing.

In Chapter 2, I tested the hypothesis that the evolution of heterostyly increased the rate at which species accumulated over time in the primrose family. In this study, I generated a densely sampled phylogeny for Primulaceae, with 265 taxa representing 36% of extant species and proportional samples of heterostylous and non-heterostylous species, to provide the most detailed picture of the evolutionary dynamics of diversification in Primulaceae to date. I demonstrated a robust association between the evolution of heterostyly and accelerated species diversification and attribute this to lower extinction, rather than higher speciation in the heterostylous clade. Additionally, I found that the gains and losses of heterostyly have different impacts on the pattern of lineage diversification over short and long evolutionary times. Jointly, the findings are interpreted as evidence for long-term beneficial effects of outcrossing.

In Chapter 3, I focused on trait diversification rather than lineage diversification. The shift from outcrossing to increased selfing is typically associated with changes in multiple floral characters, termed the selfing syndrome, notably including a reduction of floral size. I used recently developed comparative methods to study quantitative effects of the transition from heterostyly to homostyly on four floral traits among 126 Primrose species. Contrary to expectations, I found similar variability among heterostylous and homostylous flowers, but contrasting patterns among traits: homostylous flowers are smaller in some but not all respects. Patterns in trait evolution are best explained by a marked increase in the intensity of stochastic fluctuations of evolutionary trajectories associated with

losing heterostyly - an unexpected result. These results are congruent with an increased importance of drift for evolutionary trajectories of floral morphology after the loss of self-incompatibility.

In Chapter 4, I studied the reproductive implications of variation in floral morphology within the alpine, homostylous species *Primula halleri.* Unreliable pollinator service is thought to promote the evolution of self-compatible plant breeding systems, such as homostyly, because selfing may provide reproductive assurance when outcrossing opportunity is limited, e.g. in alpine habitat. However, male and female sexual organs of homostylous species may display spatial separation (herkogamy), an arrangement presumed to promote outcrossing. Extensive pollination experiments and morphological investigations revealed that herkogamy decreases during flowering, but the ultimate expression of herkogamy in mature flowers differs among individuals and populations. Pollinator-exclusion experiments indicate that herkogamy reduces a plant's potential for autonomous selfing, and emasculation and open-pollination treatments demonstrate that herkogamy markedly decreases total seed set and the potential for reproductive assurance. This study suggests that even small amounts of herkogamy can have large effects on the reproductive strategy of homostylous species, by enabling more outcrossing than generally thought to be typical of homostyly.

In Chapter 5, I investigated the evolutionary relationships among the seven species in *Primula* section *Primula*, which include well-known European species, Cowslip, Oxslip and Primrose. I analyzed a dataset of nuclear and chloroplast loci that included all species and subspecies and geographically representatively sampled, using multiple Bayesian methods for gene tree and species tree inference. In particular, I investigated the effect of misspecifying the prior on branch lengths, which results in an often overlooked phylogenetic artifact. Prior misspecification resulted in trees that were up to two orders of magnitude to long, but topological relationships were not affected. These relationships indicated extreme levels of genetic heterogeneity within species and high levels of species non-monophyly, especially in *Primula elatior*. The patterns observed in the gene trees are congruent with the interpretation that *P. elatior* in its current circumscription may represent the disjointed remnant of an ancestral species from which the other recognized species diverged.

The research in this thesis illustrates the potency of an integrated evolutionary approach, by making, wherever appropriate, connections between ecology and macroevolution. Study of ecological functioning of traits and phylogenetic patterns can be combined such that they mutually reinforce each other, contributing to the overarching goal of elucidating what processes during the history of Primulaceae have affected their diversification and what general lessons we can draw for angiosperm diversification and the evolution of plant reproductive diversity.

CHAPTER I: GENERAL INTRODUCTION

The Tree of Life is Asymmetric

Evolutionary biology is motivated by an innate desire to understand the natural world in which we live. The diversity of flowering plants represents one of the extraordinary outcomes of the evolutionary process. The most recent common ancestor of all seed plants gave rise to two lineages that persisted to the present (Magallon 2010; Smith et al. 2010): flowering plants (angiosperms), with an estimated total of 352,000 + perhaps 15% species, and all other seed plants, the gymnosperms, with only ca. 1'050 extant species, 250-350 times less than angiosperms (Joppa et al. 2010; Scheffers et al. 2012). The rapid radiation of angiosperms since the Cretaceous apparent in the fossil record (Crepet 2008; Doyle & Endress 2010) and their extreme extant diversity compared to other plants (Fiz-Palacios et al. 2011; Scheffers et al. 2012) has puzzled naturalists since pre-Darwinian times, but causes remain contentious (Crepet & Niklas 2009; Vamosi & Vamosi 2011; Crisp & Cook 2011).

The diversity of angiosperms vs. gymnosperms is a good example for the widespread phenomenon of differential diversification throughout the Tree of Life, where sister lineages differ strongly in the number of species, despite having had equal amounts of evolutionary time to diversify. For instance, this pattern has been documented recently among jawed vertebrates (Alfaro et al., 2009), flies (Wiegmann et al. 2011), cyanobacteria (Schirrmeister et al., submitted), yet our understanding of how asymmetries in diversity evolved remains fragmentary (Scotland & Sanderson 2004). Therefore, by improving our understanding of the evolutionary dynamics that affect flowering plant diversity, we may well learn about the fundamental principles that shape the pattern of diversity of life through time.

Early ideas about angiosperm diversification focused on single causal explanations based around one or more of the unique innovations, such as closed carpels, vessels, or increased growth rate,

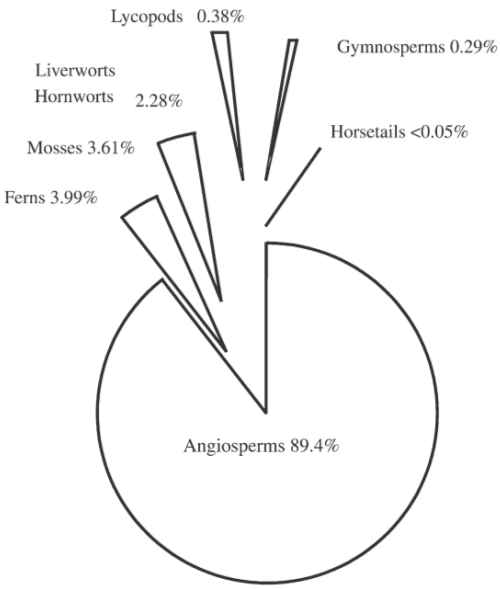


Fig. 1. Differential diversification of major groups of land plants. Although angiosperms are, by definition, the same age as their sister group, gymnosperms, angiosperms contain >250 times as many species. Image from Crepet & Niklas (2009).

shared by all angiosperms. This hypothesis has been superseded, not least because the rate at which species accumulate in a clade through time (the diversification rate, or net diversification rate, i.e., speciation rate minus extinction rate) did not shift at the base of the angiosperms (Sanderson & Donoghue, 1994). Rather, the diversification rate likely shifted several dozen times among angiosperm lineages (Smith et al. 2010). These shifts are apparently rather evenly distributed through time and in a labile pattern across lineages (Magallón & Sanderson 2001; Davies et al. 2004; Smith et al. 2011). This is in line with the sustained high rates of origination, low rates of extinction and high net diversification through time observed in the angiosperm fossil record (Crepet & Niklas 2009).

Because species arise via speciation and disappear via extinction, differential diversification necessarily results from different numbers of speciation and/or extinction events through time in either sister clade. Thus, to understand the causes of differential diversification, it is necessary to uncover the factors that affect the rate at which speciation and extinction occur. Traditionally, such factors affecting angiosperm diversification have been grouped as either intrinsic properties of species or extrinsic factors from the environment (Vamosi and Vamosi 2011). A wide range of intrinsic traits have been proposed to drive rapid diversification, including bilaterally symmetric flowers, biotic pollination, fleshy fruits, biotic dispersal, herbaceousness, polyploidy, latex canals, hermaphroditism, and self-incompatibility (reviewed by Vamosi & Vamosi 2011). Extrinsic factors include tropical habitat, available area, geographic extent of constituent species, and the time available to diversify (Vamosi & Vamosi 2011). However, it has recently been pointed out that extrinsic and intrinsic factors may interact, in other words, that diversification is about being at the right time in the right place with the right set of traits (Wagner et al. 2012). Thus, Van der Niet & Johnson (2012) concluded that "*lineage diversification depends on both intrinsic factors that determine the variation and constraints upon which selection operates, and extrinsic factors that provide the selective regime*" (Van der Niet & Johnson 2012; Vamosi & Vamosi 2011).

Reproductive systems and diversification rates

The tremendous diversity of flowering plants is especially evident in the variety of flowers, inflorescences and reproductive strategies. It is a long-standing idea that the diversity and evolutionary success of angiosperms relate to the existence of the flower, the defining feature of angiosperms (Grant 1949, Sanderson & Donoghue 1994, Crane et al. 1995, Crepet & Niklas 2009). In other words, the existence of flowers may have provided mechanisms that drove the diversification rate (reviewed in Van der Niet and Johnson 2012), for instance due to pollinator specialization (Fenster et al. 2004; Sargent 2004). However, there is an additional, fundamental dimension to the role of flowers in diversification, which lies in population genetics.

Central to the flow of genes within and between populations is the pattern of mating. A plant's mating system is its participation in fertilization as maternal and/or paternal parent, including the incidence of self- versus cross-fertilization, the diversity of outcrossed mates and their characteristics (e.g. assortative vs. disassortative mating) (definition by Harder & Barrett 2006a, p. 181). A mating system can be affected by a population's sexual system, that is, by the qualitative differences among

flowers within and between plants in the production of pollen and ovules and compatbility / incompatibility status (definition by Harder & Barrett 2006a, p. 181). Floral traits influence the flow of gametes in a population, for instance via affecting how pollinators interact with flowers, and thus affecting patterns of mating. Unlike, for instance, most animals, plants are remarkably diverse in sexual systems, offering a wealth of case studies for the evolution of reproductive strategies.

Flowers may have important effects on the course of diversification for the type of mating they enable. Especially relevant is the transition from outcrossing to selfing, which is one of the most common transitions in flowering plant evolution (Stebbins 1950; Grant 1981). Selfing is relatively "easy" to evolve: once a mutation occurs that enforces an outcrosser to become a selfer, that mutation is spread both through male and female function, giving the mutation a "two-fold transmission advantage" (Fisher 1941). Unless inbreeding depression, the reduced fitness of selfed compared to outcrossed offspring, is >0.5, such mutation will spread in an idealized population (Fisher 1941). Secondly, selfing provides an ecological advantage by avoiding dependence on vectors to transport pollen (i.e. reproductive assurance). However, selfing also brings genetic disadvantages. It decreases the extent to which genetic variation can be maintained in a population, decreases heterozygosity of individuals, promotes the expression of deleterious recessive alleles and the accumulation of deleterious mutations (reviewed in Uyenoyama et al. 1993). These negative effects may cause a poorer adaptability, compromising long-term evolutionary survival. A long-standing hypothesis, therefore, holds that the negative effects of selfing outweigh its benefits over long evolutionary times (Stebbins 1957, 1974; Grant 1958): the "dead-end"-hypothesis. Support for the "dead-end" hypothesis was, for instance, found in a phylogenetic study of Solanaceae, where self-compatible lineages were found to have high species-turnover rates and negative net diversification rates (i.e. extinction exceeded speciation; Goldberg et al. 2010). However, the causal links on how floral traits and sexual systems affect mating, how mating affects population genetic processes and how population genetic processes affect the rates of speciation and extinction, and, finally, how all this feeds back into the evolutionary trajectory of floral traits is all relatively poorly understood.

To fill in the holes in our fragmentary understanding of the general expected relationships between floral morphology, patterns of mating, and ultimately patterns of diversification, it is useful to select a model system that displays several key characteristics of the transitions described above. In this thesis I primarily studied the evolutionary dynamics associated with "heterostyly". Heterostyly is a genetic polymorphism in which plant populations are composed of two (distyly) or three (tristyly) morphs that differ reciprocally in the position of anthers and stigmas in flowers (definition by Barrett 1992, p. 1). The polymorphism in stigma and anther position, termed reciprocal herkogamy, is usually associated with a self- and intra-morph incompatibility system, and often with ancillary polymorphic characters (reviewed by Ernst 1962; Ganders 1979; Barrett 1992; Barrett & Shore 2008; Cohen 2010; Keller et al. 2012). Heterostyly is a particularly useful model system for the evolutionary dynamics of plant reproduction, because many heterostylous plant groups include homostylous species, that is, species that lost the polymorphism and became self-compatible. The function, genetics, and evolution of heterostyly is discussed further down.

Integrating ecology and macro-evolution

In this thesis, I attempt to improve understanding of the evolution of plant reproductive diversity, by applying ecological and phylogenetic approaches such that they can mutually reinforce each other. The identification of macro-evolutionary predictions from plant-functional considerations builds on a Darwinian tradition. For instance, Darwin's studies on the function of elaborate floral traits that prevent orchids from self-fertilizing led him to famously suggest that "*Nature thus tells us (...) that she abhors perpetual self-fertilization*" (Darwin 1862, p.359), implicitly predicting that few self-fertilizing species exist and those that do should not represent major evolutionary lineages. However, discussions with Hermann Müller, a pioneer of the study of floral evolution (e.g. Müller 1873, 1881), prompted Darwin to more broadly survey the occurrence of self-fertilization across many groups of distantly related plants, leading him to correct himself by stating (Darwin 1876, p. 8): "*If the word perpetual had been omitted, the aphorism would have been false. As it stands, I believe that it is true, though perhaps rather too strongly expressed.*" (Seward 2006). This example illustrates the fruitfulness of formulating macro-evolutionary predictions from functional considerations, and then using the wealth of extant diversity as a "natural experiment" to test the predictions. In this particular case, the integration of macro- and micro-evolutionary thinking allowed Darwin to identify an important pattern of variation in plant-reproductive evolution, namely that of complete versus intermediate selfing (Goodwillie et al. 2005).

A century after Darwin, David G. Lloyd arrived at a similar integration of plant reproductive function and macro-evolutionary predictions (Barrett & Harder 2006b). Baker (e.g. 1955) and Ornduff (e.g. 1969) had contributed similar ideas, but Lloyd demonstrated his arguments mathematically. As an example, Lloyd (1984) noted a generally strong bias in resource allocation within flowers towards female function, and he suggested that this was best explained by an upper limit on paternal fitness. Therefore, he reasoned, a selective force should exist to increase the proficiency of pollen transfer in terms of its precision, on the basis of which he predicted a macro-evolutionary tendency toward reduction of the number of floral parts, their fusion, and bilateral symmetry, as these traits should promote precise pollen transfer (Lloyd 1984 p.300; Barrett & Lloyd 2006b). These macro-evolutionary trends that were predicted from mathematical modelling of plant reproduction have indeed been identified across the whole of flowering plants (e.g. Endress 2011), demonstrating the potency of this approach.

Heterostyly as study object

The function of heterostyly

Heterostyly was reputedly already observed by Clusius (Van Dijk 1943, cited in Ornduff 1992). However, early botanists regarded it as "mere variability" until the mid nineteenth century, when Darwin (1862) and Hildebrand (1863) published their first extensive accounts on the "remarkable sexual relationships" of *Primula* species, emphasizing the functional importance of the floral

polymorphism. Darwin (1877) devoted most of his book "*On the different forms of flowers on plants of the same species*" on heterostyly in *Primula*, through which he promoted a functional perspective on the remarkable appearance of heterostylous flowers.

Darwin (1877) argued that the function of heterostyly consisted of two rather distinct aspects. First, the reciprocal position of sexual organs within flowers should function as a mechanical device for promoting insect-mediated cross-pollination. Pollen gets predominantly deposited on a particular part of a pollinator's body upon probing the pollen-donating flower; when the pollinator probes a second flower, the pollen primarily will be deposited on a stigma that closely matches the position of the anther in the first flower (reviewed by Keller et al. 2012). Secondly, Darwin noted that only crosses between sexual organs placed at the same position in the flowers (both low, or both high) showed full fertility. These he termed "legitimate unions", whereas crosses between high and low organs, either within the same flower, within the same plant or between different plants, were termed "illegitimate unions" (Fig. 2). Darwin, very well aware of the negative consequences of inbreeding (see above), understood that pollen grains should not be wasted in "illegitimate unions", and argued that the reciprocal position, which should promote legitimate pollen transfer, therefore also prevented pollen wastage. This view was widely accepted by later workers (Dulberger 1992).

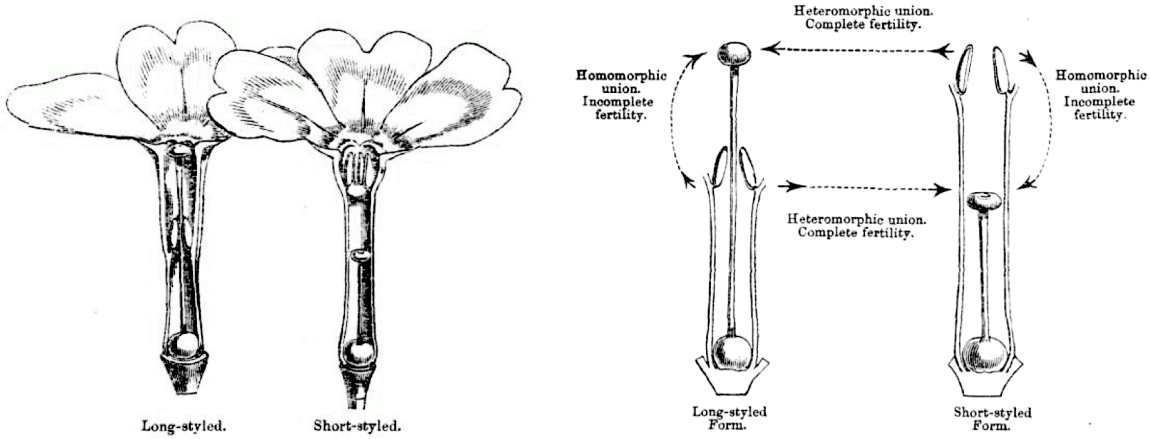


Fig 2. Heterostyly illustrated by Darwin (1862). The morphological condition that prompted Darwin to investigate its function is illustrated on the left. Darwin's investigations led him to conclude that fertilizations between morphs show full fertility ("heteromorphic unions") and self-fertilizations usually result in incomplete fertility (right illustration).

To the important functional aspects of heterostyly already discussed by Darwin (avoidance of selfing, promotion of cross-pollination, prevention of pollen wastage), Lloyd & Yates (1982, p. 904, cited in Harder & Barrett 2006b) added an important additional dimension, by discovering the mechanism of self-interference, which also applies to the function of heterostyly (Barrett 2002). Self-interference is the situation where a hermaphrodite's male and female sexual functions compete with each other. As an example, if a pollen grain lands on an incompatible stigma within the same flower, then that pollen grain is wasted and the male function compromised, but it may also compromise female function: less space on the stigma remains for other pollen grains to land. If the stigmatic surface is covered with incompatible pollen, it is easy to see that female function is compromised.

Likewise, pollen export, and thus male fitness, may be affected by the position of the stigma, for instance if the position of the style prevents a pollinator from touching an anther. In other words, the architecture of a flower that maximizes male fitness may be very different from an architecture maximizing female fitness (Johnston et al. 2009). Heterostyly may provide a way of resolving this sexual conflict (Barrett 2002), by means of spatially separating sexual organs (Lloyd & Webb 1986). Thus, heterostyly serves multiple functions: it avoids sexual interference by spatially separating sexual organs within flowers, by doing this reciprocally between morphs it promotes inter-morph pollen transport, and by having an intra-morph self-incompatibility system it further prevents selfing.

The genetics of heterostyly

In 1905, only five years after the rediscovery of Mendel's work, Bateson and Gregory published the first account on the genetics of heterostyly in *Primula sinensis* (Bateson also was the one to first coin the term genetics, around that same time), and showed that its inheritance fitted well with Mendelian principles. By crossing and self-fertilizing pins and thrums, they could establish that pins were homozygote recessive ss, and thrums were heterozygote Ss: self-fertilized thrums yield pins and thrums and must thus be heterozygote, whereas self-fertilized pins only yield pins and must be homozygote. Pins could not be homozygous dominant, because crosses of pins and thrums yield a ~50-50% mixture of pins and thrums (Bateson & Gregory 1905). The reason their work was so succesful relates to the relatively high intra-morph self-compatibility of *Primula sinensis*; their attempts to repeat the experiments in *P. vulgaris* were less successful, as they had greater difficulty in obtaining self-fertilized offspring (Ornduff 1992).

Later researchers subsequently confirmed that heterostyly in most investigated systems was also congruent with a simple Mendelian single locus, di-allelic regulation, but exceptions were also found. For instance, in *Narcussus* (Amaryllidaceae) and *Anchusa* (Boraginaceae) multiallelic incompatibility occurs, and in some species of *Amsinckia* and *Cryptantha* (Boraginaceae), and *Eichornia* (Pontederiaceae), reciprocal herkogamy occurs without a self-incompatibility system (examples cited from Dulberger 1992; topic reviewed by Ganders 1979; Lewis & Jones 1992).

Details on the genetics of heterostyly were elaborated by Alfred Ernst (Zürich, 1875 - 1968), whose publishing life spanned 61 years. Through very extensive crossing experiments that were meticulously reported, involving literally tens of thousands of manual pollinations within and between species, Ernst observed that not one but multiple loci must be involved in the inheritance of heterostyly. Specifically, he discovered that there were three loci that jointly determined the phenotye: G, the length of the style, the stigmatic papillae type and the female compatibility type, A, the position of the anthers, P, the pollen type and the male compatibility type. Thrums were heterozygous GPA/gpa; pins were gpa/gpa, congruent with Bateson & Gregory's (1905) findings. Although Ernst suggested that mutations were responsible for the occasionally observed aberrants of normal trait combinations, including homostyles, Lewis & Jones (1992) suggested, based on a re-analysis of Ernst's data, that recombination is a more likely cause for such aberrant forms to occur.

Importantly, by demonstrating that the complex trait heterostyly was built-up from several tightly linked loci, Ernst also provided a mechanism for homostyly to evolve. Homostylous plants, when having a long style, are of phenotype gPA under Ernst's model, whereas short styled plants are of genotype Gpa. This interpretation was corroborated by crossing homostylous species with closely related, heterostylous species. The prediction was that the pollen type of long homostyles (which should have the thrum male compatibility type) should still be fertile on stigmas of pin plants of closely related species. This prediction, and analogous ones, were experimentally demonstrated and referred to by Ernst in his later work as the "erweiterte Fertilitätsregel" (the extended law of fertility). After spending 30 years crossing primroses, published in thousands of pages, Ernst eventually had observed all of the eight possible phenotypes, and attempted the crosses for most of the 64 combinations (Fig. 3).

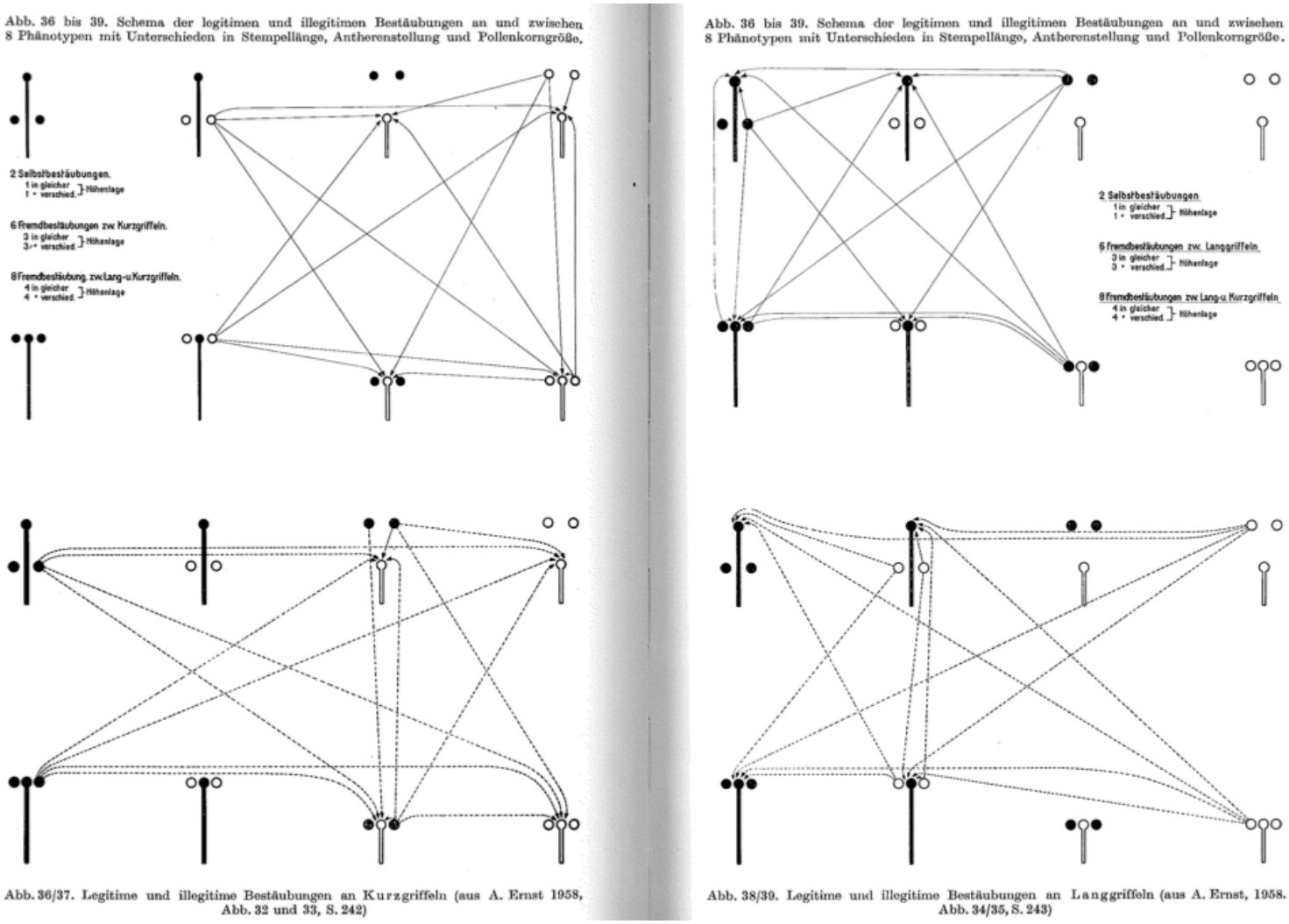


Fig. 3. The eight possible phenotypes (depicted four times) regulated by the heterostyly locus (from Ernst 1962, published at age 87) and the 64 possible crosses. According to his "extended law of fertility" (see text), crosses indicated with dashed lines are infertile, and crosses with continuous lines were fertile. During his >60 year career, Ernst experimentally demonstrated nearly all possible crosses. The GPA model is indicated by style length (G short, g long), pollen type (P black, p white) and anther position (A high position, a low position).

The evolution of heterostyly:

Given the function and genetic regulation of heterostyly, several theories have been put forward to explain the evolution of heterostyly. The two most important quantitative, genetic models are described here; older ideas are discussed by Ganders (1979) and Cohen (2010). Charlesworth & Charlesworth (1979b) described a model in which first di-allelic self-incompatibility evolved, with the morphological aspects evolving to promote outcrossing in response to "compensate" for the high inbreeding depression associated with selfing. The model was extended to incorporate the breakdown of heterostyly to homostyly (Charlesworth & Charlesworth 1979a). However, the assumption that self-incompatibility evolved first was later challenged, not least because in nature virtually no systems exist that display di-allelic self-incompatibility without morphological differentiation (the exception being some Plumbaginaceae), whereas the opposite (self-compatible species with reciprocal herkogamy) is found in several groups (Barrett 1992; Lloyd & Webb 1992a). Lloyd & Webb (1992a), therefore, designed a model in response that includes the opposite sequence of events. Here, reciprocal herkogamy would evolve first through selection for more proficient cross-pollination, and incompatibility evolves subsequently due to a combination of specialization for legitimate pollination and active selection to restrict self-fertilization (Lloyd & Webb 1992a, 1992b; Harder & Barrett 2006b). This model is still widely followed (Barrett & Shore 2008; Cohen 2010; Keller et al. 2012), and several key aspects have received support through experiments (Stone & Thompson 1994).

Primula as study system

The research in this thesis employs the family Primulaceae (primroses) as a study system, in particular the genus *Primula*. The history of the discovery of and research on *Primula* spans many centuries. Colonna (1592) suggested, according to Pax (1889), that the Greek author Dioscorides (~50-70) already referred to a *Primula* species in his "Materia Medica", but the earliest botanical descriptions of species certainly belonging to *Primula* date back to the early 16th century, in books on medicinal plants by Brunfels, Fuchs, and Bock (*P. elatior* and *P. veris*; Pax 1889). In the second half of the 16th century, Matthiolus (1558) additionally mentions *P. auricula*, which, according to Kerner (1875; cited in Pax 1889), was already in 1570 in cultivation by Holy Roman Emperor Maximillian II, in multiple varieties, along with several other species, presumably *P. farinosa* and *P. clusiana*. Clusius (1583) also knew at least ten *Primula* species. In Japan, *Primula sieboldii* has been bred as a traditional garden herb at least since the early Edo period (1603-1867), and more than 300 cultivars are known (Honjo et al. 2008).

In the 259 years since Linnaeus (1753) described the first 7 *Primula* species using the scientific binomial, 1356 taxonomic names at the specific level have been published in the genus (www.ipni.org; genus *Primula*, only specific names from the Index Kewensis). Yet, the last comprehensive synopsis (Richards 2003) recognized 430 species, indicating an above-average degree of synonymy in the genus (Scotland & Wortley 2003). Figure 4 illustrates the numbers of published names and accepted species in major synopses of *Primula* from Linneaus' Species Plantarum (1753) to

today. Still yet, the taxonomy of *Primula* is not yet "solved". First, species continue to be newly discovered and described nearly every year. Given the poor exploration of several potentially *Primula*-rich regions (notably the Arunachal Pradesh in NW India and Kashmir in NE Inda / Pakistan), it is quite likely species will continue to be discovered in the decades to come. Secondly, and perhaps even more importantly, there is still a lot of controversy concerning species concepts of local endemics in the Sino-Himalayan region. Of the 300 *Primula* species that the Flora of China (Hu & Kelso 1996) covers, 46* are not accepted in the same circumscription by Richards (2003), who compiled the most recent comprehensive treatment of the genus. It will be a gigantic task to provide a stable taxonomy for *Primula*, yet necessary to improve the understanding of the evolution of *Primula*. In particular, the better understood the taxonomy is, the more valuable *Primula* becomes as a model for a broad range of evolutionary studies.

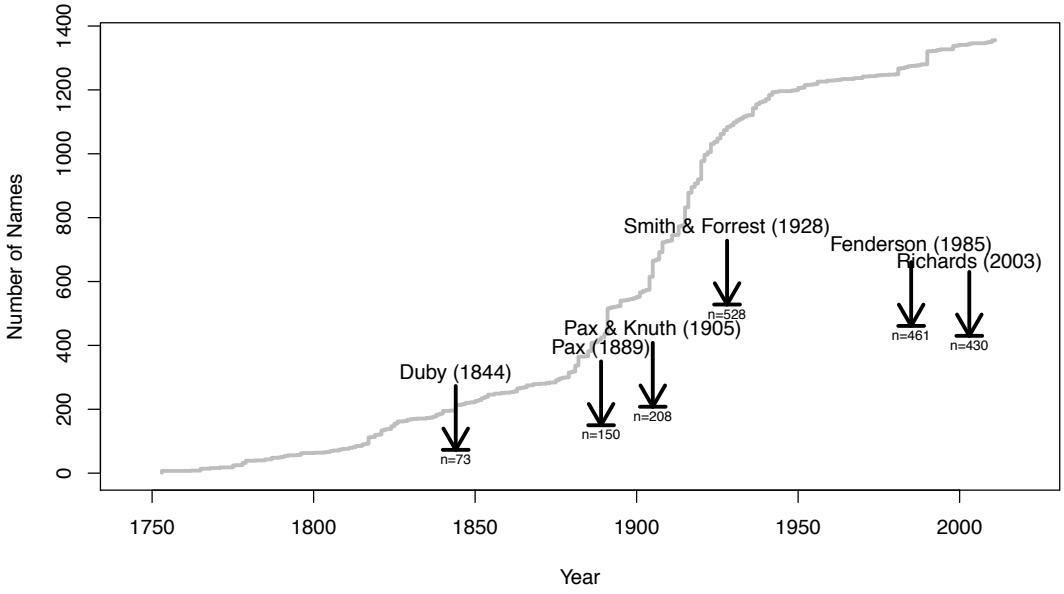
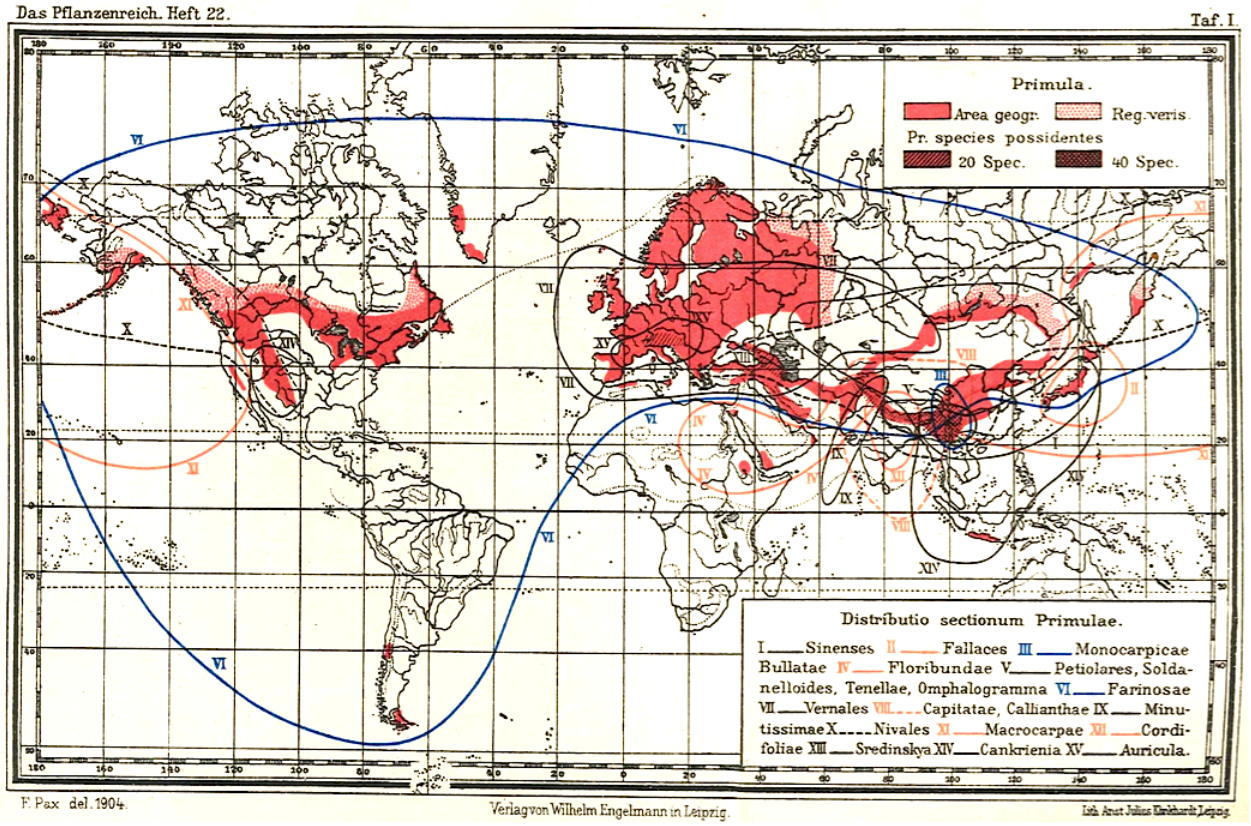


Fig 4. Cumulative number of published names in *Primula* (grey line) and the number of actually accepted species in six major, comprehensive revisions of the genus, indicating reference and number of species accepted. Data on taxonomic names extracted from the International Plant Name Index; synopses cited are listed in the reference at the end of this chapter.

Primula is one of the spectacularly rich groups in the Sino-Himalayan region (Qiu et al. 2011), where ca. 80% of its five hundred species occurs. Interestingly, the closely related genus *Androsace* has a very similar overall distribution area, and also radiated spectacularly in the same regions (Figs 5-6), yet contains four times fewer species. There are ca. 6-8 additional genera in Primulaceae, depending on taxonomic authority, all of which have considerably fewer species than *Primula* or *Androsace*. Heterostyly occurs in most species of *Primula*, in *Dionysia* (which is nested within *Primula*; Mast et al. 2006), *Hottonia palustris* and *Androsace vitaliana*. The latter species shows

*These doubtful species are: *Primula aemula, P. anisodora, P. barbatula, P. beesiana, P. chamaedoron, P. chamaethauma, P. chienii, P. chrysochlora, P. cunninghamii, P. epilosa, P. euosma, P. graminifolia, P. helodoxa, P. hoffmanniana, P. humilis, P. hypoleuca, P. knuthiana, P. kongboensis, P. laxiuscula, P. loeseneri, P. maikhaensis, P. meiotera, P. melanantha, P. melanops, P. monticola, P. neurocalyx, P. ninguida, P. obsessa, P. prattii, P. prevernalis, P. pseudoglabra, P. purdomii, P. reflexa, P. runcinata, P. saxatilis, P. scopulorum, P. sinomollis, P. sinoplantaginea, P. smithiana, P. socialis, P. strumosa, P. tardiflora, P. tayloriana, P. tenuipes, P. tsariensis, and P. wangii.*

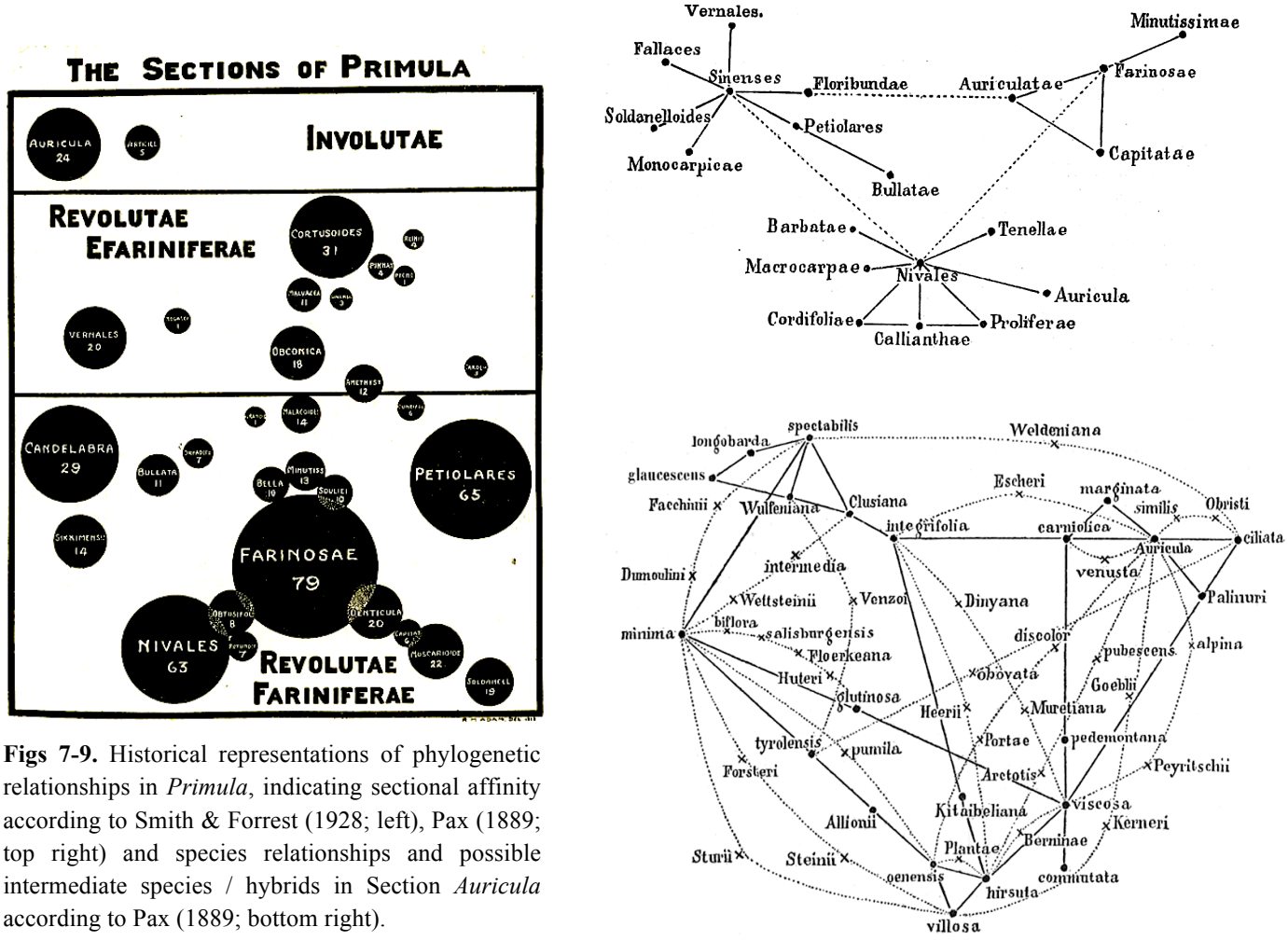
a strong stylar polymorphism but a weak anther polymorphism (pers. obs., Schaeppi 1935), therefore it appears to represent an intermediate stage in the sequence of morphological steps for the evolution of heterostyly in the model that Lloyd & Webb (1992a) proposed.



Figs 5-6. Distribution of sections recognized in *Primula* by Pax & Knuth (1905; top), and in *Androsace* (Schneeweiss et al. 2004; right). Although Pax & Knuth knew less then half of all species now known, the overall known distribution is very similar. The area in the Sino-Himalaya circled to have >40 species is now known to harbour >200 *Primula* species. Lines demarcate sectional distributions. Hatched areas in the right Figure illustrate the distribution of nested *Douglasia*; star indicates monotypic nested *Pomatosace*, dark blurry area indicates region of high diversity. Note the similarities between distributions and centres of diversity in the two Figures.

Phylogenies provide the tool that is "*necessary to think clearly about differences between species and to analyze those differences statistically*" (Felsenstein 2004). Early naturalists working on *Primula* already used "phylogeny-like" diagrams as a tool to think clearly about sectional delimitation and provide a handle on the overwhelming number of species described over the years. Usually these represented various types of diagrams to express an implicit "sense" for evolutionary affinity among species, rather than the result of some formal analysis to infer a bifurcating diagram (Figs 7-9). Therefore, these "phylogenies" did not provide a means to statistically analyze differences between species, rather, they are graphical summaries

of large amounts of anecdotal data on the structure of biological diversity (Figs 7-9). However, to statistically evaluate differences between species, an explicit, quantitative approach is needed. Then, phylogenies can be used to study character history, biogeography, diversification rates, etc, and the correlations between them. Clearly, when phylogenetic relationships among species form the framework in which evolutionary phenomena are interpreted, it is of utmost importance to estimate a phylogeny accurately, or at least to know *how* accurate the inferred phylogeny is (O'Meara 2012). In recent years, much progress has been made toward resolving the phylogeny of *Primula* based on molecular markers (e.g., Conti 2000, Mast et al. 2001, Mast et al. 2004, Mast et al. 2006, Yan et al. 2010), yet traditionally recognized sections often do not form well supported evolutionary groups (e.g. Yan et al. 2010). However, studies of character evolution have yielded valuable insights (e.g., Conti et al. 2000; Mast et al. 2004; Mast et al. 2006; Yan et al. 2010), although the power of modern phylogenetic-statistical analysis for evolutionary study of *Primula* has not been fully exploited (O'Meara 2012



Figs 7-9. Historical representations of phylogenetic relationships in *Primula*, indicating sectional affinity according to Smith & Forrest (1928; left), Pax (1889; top right) and species relationships and possible intermediate species / hybrids in Section *Auricula* according to Pax (1889; bottom right).

When Darwin (1862) studied heterostyly, only a few species were known to him. Scott (1865) provided the first attempt to comprehensively discuss the occurrence of heterostyly and homostyly in Primulaceae species, and needed only a few pages to do so. Ernst's monumental work on the breeding systems of *Primula*, conducted in collaboration with Smith and Forrest in Edinburgh (Ernst 1938),

resulted in a series of seven publications Ernst (1938, 1949, 1953, 1956, 1959, 1961, 1962) that jointly encompass ca. 835 pages. In these works, Ernst comprehensively discussed floral-morphological patterns within and between known *Primula* species, resulting in his idea that floral evolution in *Primula* is remarkably "mannigfaltig" (multifarious); more diverse than he expected himself from finding a widely valid "erweiterte Fertilitätsregel" (extended law of fertility) underpinning the shared inheritance system of heterostylous characters among *Primula* species. Surprised, and perhaps disappointed by his own conclusion, the final words that Ernst (1962) wrote at age 87 in his corpus of ca. 7,000 pages[2] were: "*Je tiefer wir in die Geheimnisse der Natur vordringen, um so kürzer erscheint uns das zurückgelegte Wegstück, um so länger der vor unserem Geiste sich auftuende, in weiter Ferne sich verlierende Weg zur Erkenntnis*" (freely translated: The further we advance into the mysteries of nature, the shorter seems the distance we covered, and the longer seems the path, appearing in our mind but loosing itself in the distance, that will lead us to understanding) .

Given the rich history of the investigations of *Primula* and heterostyly, this thesis represents a rather minor piece of progress on the long and winding "*Weg zur Erkenntnis*".

The upcoming chapters

The upcoming chapters form to some extent a logical continuation of the topics outlined above, by employing phylogenetic (Chapter 2, 3, and 5) and ecological (Chapter 4) methods to address questions related to Primulaceae diversification and the evolution of heterostyly (Chapter 2), floral evolution in *Primula* and selfing-outcrossing transitions (Chapter 3), floral morphology of *Primula halleri* and reproductive-ecological function (Chapter 4) and methodological artifacts related to Bayesian phylogeny inference in *Primula* Section *Primula* (Chapter 5). In Chapter 6 I highlight a few major findings and suggest promising avenues for further research. In these Chapters, I attempt to make, wherever appropriate, connections between ecology and macroevolution, with the overarching goal of trying to elucidate what processes during the history of Primulaceae have affected their diversification and what general lessons we can draw for angiosperm diversification and the evolution of plant reproductive diversity. The central themes of the chapters are the following questions:

- When did heterostyly evolve in Primulaceae, and do heterostylous lineages (species) diversify at a different rate than non-heterostylous lineages?
- Does the loss of heterostyly and evolution of homostyly in *Primula* affect the rate at which flowers (traits) diversify?
- Did the loss of heterostyly in *Primula halleri* result in a breeding system that allows for reproduction without pollinators?

[2] Besides being a diligent *Primula* geneticist, Ernst also worked on the re-establishment of vegetation after the vulcanic eruption of Krakatau, wrote a book on apomixis, and he was a passionate and public proponent of social Darwinism and eugenetics, at least in the 1920's (Ernst 1927).

- What are the relationships of species in *Primula* section *Primula*, and to what extent are current phylogenetic methods capable of inferring relationships among closely related species?

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CHAPTER II: CONTRASTING LONG- AND SHORT-TERM IMPACTS OF HETEROSTYLY ON EVOLUTIONARY DIVERSIFICATION RATES IN DARWIN'S PRIMROSES

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Abstract

The reasons why life is so diverse and why clades differ so substantially in rates of lineage accumulation through time across disparate branches of the Tree of Life are central questions in evolutionary biology. Here we investigate the exceptional diversity of flowering plants (angiosperms), a clade of 350,000 species that is >250x larger than its sister group, and which dominates the world's terrestrial biomes. Floral innovations, their associated pollinator syndromes and plant breeding systems, and especially their repeated evolution across multiple clades, have been proposed as the main drivers of angiosperm diversification. While the tremendous diversity of floral morphology is amply documented, the impacts of this diversity on species diversification remain poorly understood. To investigate these questions, we focus on the complex floral polymorphism "heterostyly", whereby floral morphs differ in the reciprocal placement of male and female organs enforcing outcrossing while facilitating inter-morph fertilization, which has evolved at least 20 times. Using the classical model system for heterostyly, the primrose family, we infer a time-calibrated, 265-taxon phylogeny for Primulaceae, reconstruct the evolution of heterostyly, and estimate speciation, extinction, and net diversification rates. Results show a significant acceleration in species diversification associated with the evolution of heterostyly, higher extinction rates in non-heterostylous lineages, and contrasting effects of gains and losses of heterostyly over short and long evolutionary timescales. These results demonstrate the importance of floral traits in driving diversification, in line with the idea that the ability of flowers to repeatedly reinvent themselves underpins the extraordinary evolutionary success of angiosperms.

Introduction

Explaining why life is so diverse, the processes that generate the great diversity of life forms, and how and why some lineages became more species-rich than others are central questions in evolutionary biology (Benton & Emerson, 2007; Benton, 2009; Mayr, 1982). Large differences in species numbers between sister lineages occur in disparate organismal groups across the Tree of Life, including jawed vertebrates (Alfaro et al. 2009), flies (Wiegmann et al. 2011), cyanobacteria (Schirrmeister et al. submitted), and land plants (Fiz-Palacios et al. 2011), yet our understanding of how these asymmetries in diversity evolved remains fragmentary (Scotland

& Sanderson 2004). The angiosperms (flowering plants) are one of the most successful evolutionary lineages. With ca. 223,000-352,000 species (Paton et al. 2008; Scheffers et al. 2012), flowering plants comprise 250 times more species than their sister group, the remaining seed plants, and dominate the world's terrestrial biomes and many aquatic habitats (Crane et al. 1995, Niklas 1997). Ever since Darwin (1879 cited in Darwin & Seward 1903, p.20-21) described the rapid rise and early diversification of the angiosperms as “an abominable mystery”, many theories have been proposed to explain how and why the flowering plants became so diverse and ecologically successful (Crepet 2000, Crepet & Niklas 2009; Vamosi & Vamosi 2011).

Recent studies suggest that the higher species diversity of flowering plants is explained by multiple, independent increases in rates of diversification (i.e., speciation minus extinction) since the establishment of the angiosperms in the Cretaceous (Crepet, 2008; Magallón & Sanderson, 2001; Davies et al. 2004; Smith et al. 2011), rather than by a single increase concomitant with their origin and associated with the evolution of a unique trait (Sanderson & Donoghue, 1994). The multiple episodes of diversification in different flowering plant clades are likely to have been driven both by extrinsic opportunities, e.g., climatic and geological events, as well as by the evolution of intrinsic traits, i.e. evolutionary innovations such as morphological and ecological features (Vamosi & Vamosi 2010, 2011). This “polyepisodic” view of angiosperm diversification is concordant with the idea that angiosperms are inherently more adaptable and plastic than other plants, which may allow them to "reinvent themselves" time and again (Crepet & Niklas, 2009: 377).

The spectacular diversity of flowers and inflorescences, with their associated pollination syndromes and breeding systems, epitomizes the idea of evolutionary reinvention and has long been proposed as a key explanation of angiosperm diversification (e.g. Midgeley & Bond 1991, Pellmyr 1992, Gorelick 2001, Crepet & Niklas 2009, Kay & Sargant 2009, Van der Niet & Johnson 2012). Flowers engage in intricate relations with biotic pollinating agents, prompting the evolution of pollinator specialization (Fenster et al. 2004) and reproductive isolation (Grant 1949), often ensuring sexual reproduction via outcrossing in highly dispersed populations of relatively few individuals (Burger, 1981), and allowing for efficient seed production and dispersal (Stebbins, 1981). Flowers are thus thought to facilitate speciation and/or reduce the risk of extinction, accelerating rates of species diversification and elevating species numbers in angiosperms compared to other plant lineages. The high incidence of repeated evolution of similar floral features (i.e. convergent evolution; Endress, 2011a,b) reflects the morphological plasticity and evolutionary potential of flowers.

Despite the proposed key role of flowers in angiosperm diversification, few studies have explicitly linked specific floral traits with macro-evolutionary or temporal patterns of diversification. Sargent (2004) concluded that the greater potential for pollinator-specialization in plants with bilaterally symmetrical flowers may explain their greater number of species, compared to sister groups with radially symmetrical flowers (Neal et al. 1998). Similarly, the evolution of floral nectar spurs in columbines (*Aquilegia*) and other angiosperm lineages was supported as a key innovation driving rapid species diversification (Hodges and Arnold, 1994, 1995; Wollenberg 1996). However, aside from these two well-known examples and the general notion that reproductive systems enabled by a particular set of floral traits may affect speciation and extinction probabilities (Goldberg et al. 2010; Johnson et al. 2011; Ferrer & Good 2012), the impacts of specific floral traits on rates of diversification remain largely unknown (Moore & Donoghue 2007).

To elucidate the evolutionary consequences of specialized floral syndromes,, we investigate the effects of one of the more remarkable floral innovations in angiosperms, i.e., the floral polymorphism “heterostyly”, on the trajectory of species diversification. The function of heterostyly was first elucidated by Darwin (1862, 1877)

in a series of studies on primroses (*Primula*) that led him to remark *"I do not think that anything in my scientific life has given me so much satisfaction as making out the meaning of the structure of these plants"* (Darwin 1887: 91). Heterostylous populations consist of two (distyly) or three (tristyly) genetic morphs that differ in the reciprocal placement of sexual organs between flowers: the position of the stigma in one morph corresponds to the position of anthers in the other morph, and vice versa (Fig. 1). This morphological arrangement, known as reciprocal herkogamy, promotes efficient pollen transfer between different morphs via the delivery and uptake of pollen on distinct parts of the pollinator's body and is often complemented by a physiological mechanism that prevents pollen germination on the same flower or floral morph, these two factors jointly enforcing outcrossing (Darwin 1877, Ganders 1979, Barrett 1992, Barrett & Shore 2008, Cohen 2010).

Fig. 1. Heterostyly in *Primula*. The two floral morphs in *P. farinosa*: the position of the stigma (♀) in one morph corresponds to the position of the anthers (♂) in the other morph, and vice versa (i.e., distyly).

Occurring in 199 genera distributed over 28 families in 15 orders (Naiki 2012) and with at least 20 independent evolutionary origins (Barrett 1992; Naiki 2012), heterostyly provides a potent example of convergent evolution (Scotland 2010; Wake et al. 2011), illustrative of how angiosperm flowers have repeatedly "reinvented" themselves (sensu Crepet & Niklas 2009). The recurrent origin of this complex floral polymorphism begs the question of whether it conveys an evolutionary advantage to groups possessing it, yet the impacts of heterostyly on rates of speciation and extinction remain untested. The function of heterostyly suggests that such effects can indeed be expected: promotion of precise pollen transfer could facilitate speciation driven by small changes in floral morphology that might affect reproductive isolation (Johnson 2006; Armbruster & Muchhala 2009); additionally, high outcrossing rates and self-incompatibility could buffer heterostylous lineages against extinction (Takebayashi & Morell 2001; Goldberg et al. 2010). Comparisons of species numbers between related heterostylous and non-heterostylous lineages to elucidate the role of the floral polymorphism in diversification have so far been hampered by lack of species-level phylogenies, lack of sufficient resolution in phylogenies (e.g., *Narcissus*, Graham & Barrett 2004), incomplete knowledge of the distribution of character states in relevant groups (e.g., Linaceae, McDill et al. 2009), or because the phylogenetic history of the trait is

characterized by multiple gains and/or losses among closely related species (e.g., Boraginaceae, Cohen 2012; Menyanthaceae, Tippery & Les 2011). Furthermore, sister clade comparisons cannot disentangle the possible impacts of heterostyly on speciation rates from extinction rates (Nee 1994). A detailed phylogenetic analysis is required to evaluate the macro-evolutionary effects of heterostyly.

Here we test the hypothesis that the evolution of heterostyly increased the rate at which species accumulated over time in the primroses (Primulaceae s.str., i.e., Primulaceae subfamily Primuloideae sensu APGIII, hereafter Primulaceae). The family is a strongly supported monophyletic group (e.g. Källersjö et al. 2000; Mast et al. 2001) of perennial (or occasionally annual) herbs, often with a leaf rosette. Petals are fused to form a corolla tube to which the anthers are attached by typically short filaments. The family is notable for its variation of breeding systems, including both heterostylous (i.e., distylous) and non-heterostylous taxa. Within Primulaceae, heterostyly is mainly confined to the largest genus in the family, *Primula* sensu lato (i.e. including several nested genera), a clade that is 20 times the size of its sister clade (Mast et al. 2006). To investigate the possible effects of heterostyly on diversification, we reconstruct a well-resolved and robustly supported, time-calibrated phylogeny of the Primulaceae and use recent analytical methods to evaluate the hypothesis that the evolution of heterostyly promoted diversification. Specifically, we designed an analytical pipeline (Text S1, where S denotes items in the Appendix of Supplementary Information) to test whether (i) diversification rate/s increased along the branch/es where heterostyly evolved; (ii) speciation and/or extinction rates differ significantly between heterostylous and non-heterostylous lineages; and (iii) more extant heterostylous species exist than expected from background diversification rates in non-heterostylous clades.

Results

We recovered a well-resolved phylogeny with strong support from posterior probabilities for three main clades that differed markedly in species numbers: 1) /Primula, (slashes indicate conventional clade names; Mast et al. 2001, 2006), with 190 species, 151 of which are heterostylous, 2) /Soldanella, with 9 species, one of which is heterostylous; 3) /Androsace, with 66 species, one of which is heterostylous (Fig. 2, Fig. S1); these results were in line with those of previous phylogenetic studies (Källersjö et al. 2000; Mast et al. 2001; Schneeweiss et al. 2004; Mast et al. 2006; Yan et al. 2010; Boucher et al. 2012). These three main clades are each most species-rich in the Sino-Himalayan region and are widely distributed in the montane-alpine zones throughout the northern hemisphere. The split of /Primula from /Androsace (i.e., root node in Fig. 2) was dated at 38.825 MYA, with a 95% credibility interval (58.51 - 20.51 MYA) that overlaps with previous divergence time estimates for that node (Yesson et al. 2009; Text S1).

Results from the Bayesian, maximum likelihood and parsimony character-state reconstructions all indicated that heterostyly evolved early in the history of Primulaceae (Table 1; Table S1) and confirm that the most recent common ancestor of all members of /Primula was heterostylous (Mast et al. 2006). The single origin of heterostyly along the stem lineage of /Primula (Fig. 2, node c) was followed by several, deeply nested losses. However, the Bayesian and Maximum Likelihood reconstructions also suggested that heterostyly might have evolved earlier, with additional losses along the stem lineages of /Androsace and/or /Soldanella, but BayesFactors did not significantly favor that scenario over the most parsimonious reconstruction described above (Table 1; Table S1). While heterostyly is supported to have evolved independently in *Androsace vitaliana* (/Androsace) and *Hottonia palustris* (/Soldanella; Fig. 2), in those taxa the trait differs morphologically from the typical heterostyly of /Primula (Schaeppi 1934, 1935; see Text S1).

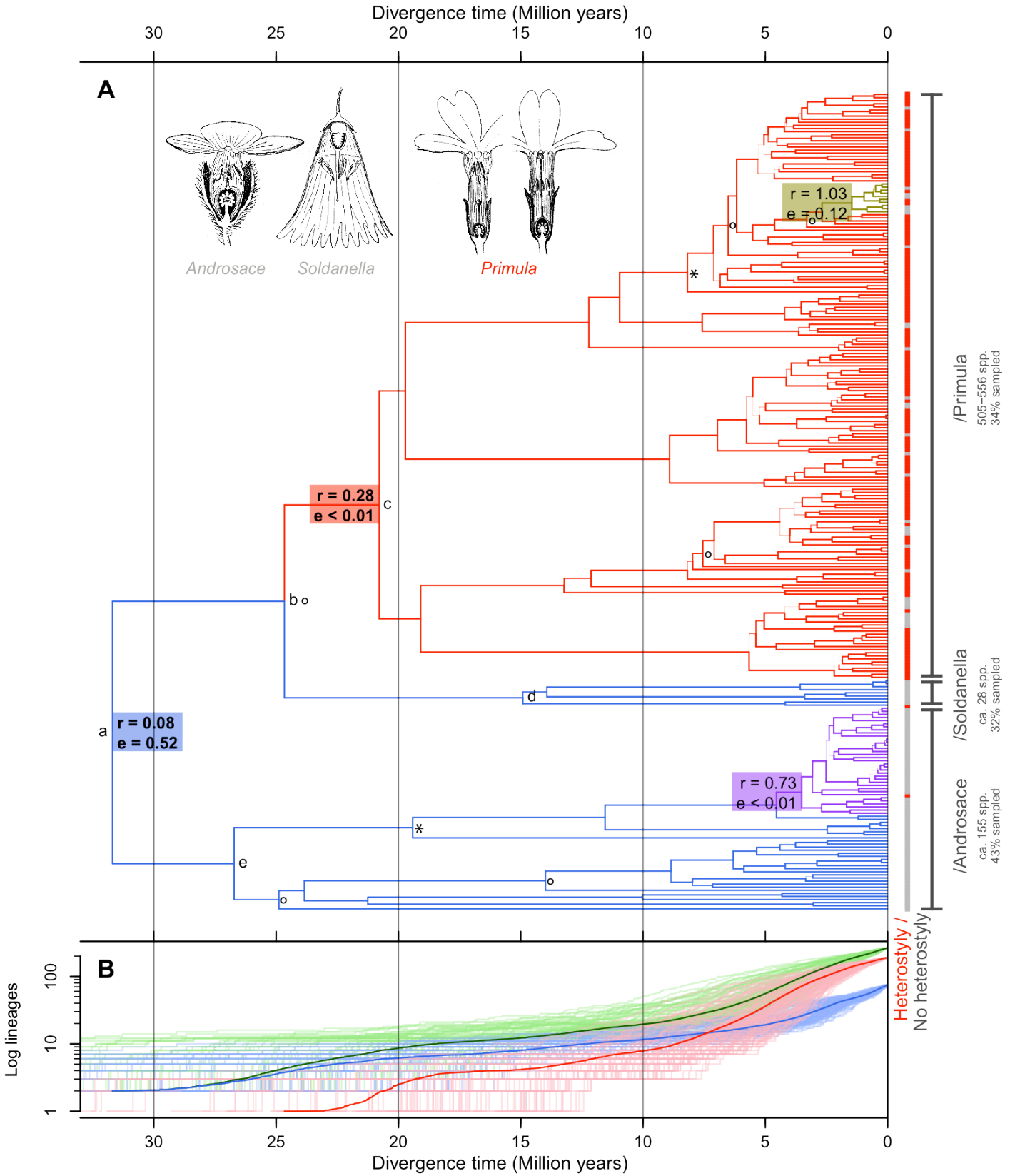

Fig. 2. Time-calibrated, 265-taxon phylogeny of Primulaceae with associated semi-logarithmic Lineage Through Time (LTT) plots. (A) Maximum clade-credibility chronogram (inferred from UnCorrelated LogNormal, i.e., UCLN, dating analysis) and diversification rates in Primulaceae. The rightmost vertical bar indicates the major clades, with total numbers of species and percentages of sampled species; the second vertical bar indicates character coding according to scheme 2 (see Methods): red=heterosyly; grey=no heterostyly. Ancestral character states were reconstructed for the five, labeled

nodes (see Table 1). Branch colors designate tree partitions inferred to evolve at four significantly different rates by Medusa (Alfaro et al. 2009), with Maximum Likelihood estimates of net diversification rate (*r*) and extinction fraction (*e*) for each tree partition. Stars ($p<0.05$) and circles ($0.05<p<0.1$) at nodes denote significant shifts to higher diversification rates in the more species-rich daughter lineage inferred by SymmeTree (Chan & Moore 2004). Branch thickness is proportional to posterior probability. Floral sketches are modified from Schröter (1908). For divergence time estimation, nodes a and b were constrained (see methods); confidence intervals for divergence times of all nodes under different analytical scenarios are provided in SI Appendix, Fig. S1BC). (B) LTT plots derived from the time-calibrated phylogeny, indicating the number of lineages through time in the heterostylous clade /Primula (red), the paraphyletic grade /Soldanella + /Androsace (blue), and all clades (green). Transparent lines designate individual LTT plots calculated from each of 100 trees randomly drawn from the posterior distribution of chronograms. Thick lines indicate the average number of lineages through time across the trees, calculated with TreeSim (Stadler 2012) in R.

Table 1. Origin of heterostyly inferred from Bayesian, Maximum likelihood (BayesTraits) and Maximum Parsimony (Mesquite) analyses, based on the tree of Fig. 2 and character coding scheme 2. Most parsimonious reconstruction: values indicate that heterostyly either did (1) or did not evolve (0) at the node; Maximum likelihood reconstruction: values indicate the proportion of likelihood associate with the node being heterostylous; Bayesian reconstruction: values represent the 95% intervals of highest posterior density for the probability of nodes being heterostylous. BayesFactor: values are natural log BayesFactors, where > 2.3 and < -2.3 represent significant support for heterostyly or no heterostyly, respectively, at the node. See Table Sx for results based on different coding schemes and dating analyses.

Node of interest	Most parsimonious reconstruction	Maximum likelihood reconstruction	Bayesian reconstruction	BayesFactor
a	0	0.49	0.19-0.81	0.09
b	0	0.89	0.70-1.00	1.55
c	1	1.00	0.96-1.00	7.44
d	0	0.20	0.05-0.40	-1.68
e	0	0.06	0.01-0.18	-3.88

Four tree partitions with significantly different diversification rates were identified in the Primulaceae tree using Medusa (Fig. 2, Table S2A; Alfaro et al. 2009). Notably, the analyses detected a 2.18-3.46 fold increase in diversification rate associated with the evolution of heterostyly (see root branch and b-c: Fig. 2). The variation among estimates of the magnitude of the rate change depended on the tree-reconstruction methods used (Table S2A). The shift in the rate of species diversification along branch b-c is robust to phylogenetic uncertainty, being recovered in each of the 100 trees sampled from the posterior distribution of phylogeny estimates. Congruently, semi-logarithmic lineage-through-time plots indicated a steeper average slope and thus a higher rate of lineage accumulation for the heterostylous clade /Primula than for the paraphyletic grade of /Androsace and /Soldanella (Fig. 2). A diversification-rate change along the same branch was also recovered by a topology-based analysis using SymmeTree (Chan and Moore 2004), albeit with marginal significance ($p=0.06$; Table S2B). SymmeTree identified several additional rate shifts, but none were congruent with the Medusa analysis or associated with the transition to heterostyly (see above; Fig. 2, Table S2B).

Clade-based analyses using BayesRate (Silvestro et al. 2011) estimated BayesFactors that strongly supported a diversification model with lower extinction rates in the heterostylous clade (i.e. /Primula) than in the non-heterostylous clades (/Soldanella + /Androsace) and speciation rates that did not significantly differ between the two tree partitions, leading to higher net diversification rates in the heterostylous clade (Fig. 3A; Table 2; Fig

S2). Character-state-based analyses using BiSSE (Maddison et al 2007; FitzJohn et al. 2009), accounting for uncertainty in the identification of the branch associated with the origin of heterostyly and the fact that not all species in /Primula are heterostylous, also suggest that the extinction rate associated with heterostyly is lower than that associated with no heterostyly and that speciation rates did not differ between the character states, with lower extinction again explaining the higher net diversification rate associated with heterostyly (Fig. 3B; Fig. S2). Taken together, the results from the Medusa, SymmeTree, BayesRate and BiSSE analyses all supported an increase in net diversification rate along the branch where heterostyly evolved (hypothesis i) and a significant difference in extinction rates between heterostylous and non-heterostylous lineages (hypothesis ii).

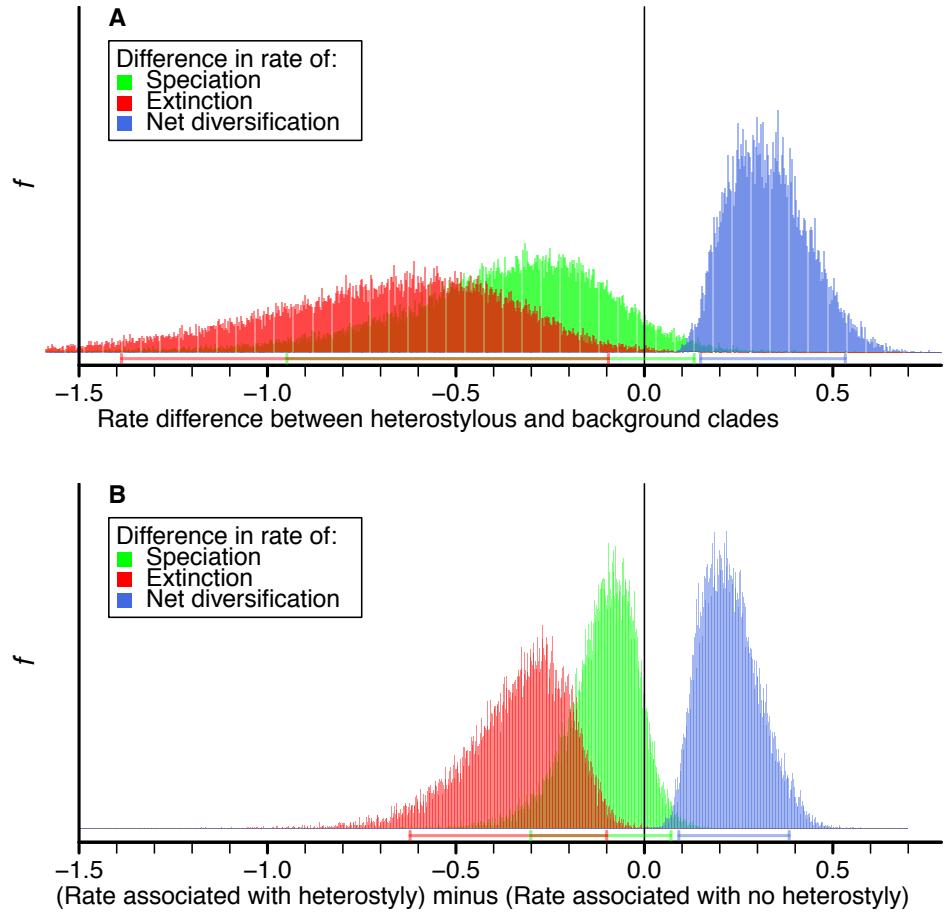

Fig. 3. Frequency distributions of differences in rates of extinction (red), speciation (green), and diversification (blue) between (A) heterostylous and non-heterostylous tree partitions using BayesRate (Silvestro et al. 2011) and (B) heteorstylous and non-heterostylous lineages using BiSSE (Maddison et al. 2007; FitzJohn et al. 2009), based on the tree of Fig. 2. Lines of corresponding colors below the distributions denote the 95% intervals of highest posterior density; intervals that include zero indicate no significant difference in rate. Both types of analyses indicate no significant difference in speciation rates between between heterostylous and non-heterostylous lineages, but significantly lower extinction rates, hence, higher diversification rates of heterostylous lineages.

Table 2. Results of BayesRate analysis based on the tree of Figure 2. Left-most column shows the diversification models considered, indicating whether the heterostylous and non-heterostylous clades received a clade-specific parameter for speciation and/or extinction rate or were modeled with one, global parameter. The number of free parameters, the log marginal likelihood, and the relative support as BayesFactor is also indicated for each diversification model. BayesFactors are reported for the pairwise comparison with the model that received the highest marginal likelihood, where values < -2.3 indicate that the fit to data is significantly worse than under the best model.

Diversification model	Number of parameters	Marginal Likelihood	BayesFactor support relative to best model
Global speciation, global extinction	2	-674.63	-23.15
Global speciation, clade-specific extinction*	3	-663.06	0.00
Clade-specific speciation, global extinction	3	-709.96	-93.80
Clade-specific speciation, clade-specific extinction	4	-717.91	-109.70

Notes: * This model provides the best fit to the data.

Frequency distributions of the number of species expected in the /Primula clade estimated under the background diversification rate of non-heterostylous clades were significantly lower than the diversity observed in the focal clade (Fig. 4), irrespective of corrections for unsampled taxa, non-zero extinction, and whether the origin of heterostyly occurred at the stem (see node c, Fig. 2) or the crown node of /Primula (node d; see Fig. S3). These results suggest that more heterostylous species exist than expected from background diversification rates in non-heterostylous taxa (hypothesis iii).

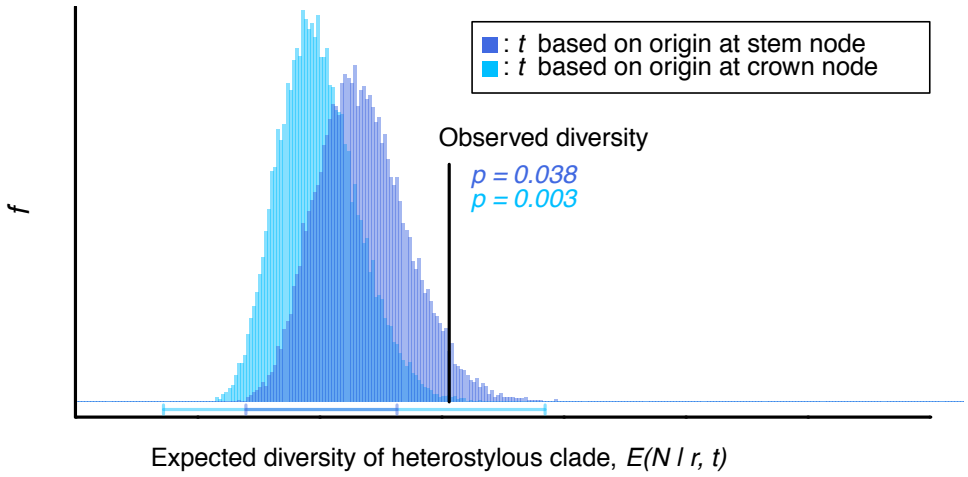

Fig. 4. Frequency distributions of species diversity in the /Primula clade expected from the background diversification rate, estimated from a separate BEAST run in which the root age was corrected, and the time since the origin of heterostyly at the stem (b) or crown (c) nodes, respectively (see tree of Fig. 2), indicating observed diversity and associated p-values. The observed diversity of the /Primula clade is significantly higher than expected (see SI Appendix, Fig. S3 for details).

Discussion

The existence and diversity of flowers and their relationships with biotic pollinators have often been suggested to be key factors underpinning angiosperm diversification (Van der Niet & Johnson 2012). Nevertheless, phylogenetic evidence that the evolution of particular floral traits, or in fact any other morphological traits, may drive high diversification rates of plants is surprisingly rare (Moore & Donoghue 2007; Van der Niet & Johnson 2012), although there are some notable exceptions, e.g. zygomorphy (Sargent 2004), nectar spurs (Hodges & Arnold 1994, 1995, Wollenberg et al. 1996), extra-floral nectaries (Marazzi & Sanderson 2010) and succulence (Klak et al. 2004, Arakaki et al. 2011). In this study, we generated a densely sampled phylogeny for Primulaceae (Fig. 2), with 265 taxa representing 36% of extant species and proportional samples of heterostylous and non-heterostylous species (Table S4), to provide the most detailed picture of the evolutionary dynamics of diversification in Primulaceae to date. We demonstrate a robust association between the evolution of heterostyly and accelerated species diversification in Primroses (Fig. 1) and attribute this to lower extinction, rather than higher speciation in the heterostylous clade (Figs 2-4, Table 2). At every step of our analytical pipeline (Text S1), we account for uncertainty associated with phylogenetic analyses and divergence time estimation. For instance, we account for our observation that different "relaxed clock" models may have a marked effect on the overall tree shape, by performing all diversification-rate analyses using trees that were estimated under two different models for subsitution-rate variation among branches (i.e. UCLN and UCEXP; Table S2 and Figs S1-S3). We also fully account for large confidence intervals on branch lengths stemming from the low number of available fossil calibrations (Fig. S1BC), suggesting that results are robust to phylogenetic uncertainty.

Heterostyly increases diversification rates in Primulaceae

Floral traits implied as drivers of high rates of diversification are commonly associated with pollinator shifts, thereby providing effective barriers to gene flow and prompting speciation (Grant 1949; Hodges & Arnold 1995; Johnson 2006; Whittall & Hodges 2007; Van der Niet & Johnson 2012), but two observations suggest heterostyly functions differently in Primulaceae. First, an important underlying assumption of the hypothesis that pollinator shifts can explain high diversification rates is that flowers have a high degree of pollinator specialization (i.e. are visited by very few pollinating species) and that pollinator shifts are frequent during clade diversification (Van der Niet & Johnson 2012). However, most heterostylous Primulaceae have a generalized-hymenopteran pollination syndrome and are thus likely to be pollinated by a suite of species, making speciation via pollinator shifts unlikely (Waser 1998). Secondly, the BiSSE and BayesRate analyses provide no evidence for an elevated speciation rate associated with heterostyly. Rather, they suggest that the high diversification rate of heterostylous lineages is due to a decrease in extinction rate following the evolution of heterostyly (Fig. 2; Fig. S2). Although the accuracy of *absolute* extinction rate estimates from phylogenies is contentious, especially when diversification rates vary among lineages (Rabosky 2010), our results show strong congruence among methods that rely on fundamentally different likelihood calculations (Figs 3A vs. 3B, Figs S2A vs. S2B) and partially correct for non-constancy of diversification rates by inferring clade-specific or state-dependent diversification rates. Furthermore, we infer a difference between extinction rates, rather than emphasizing any absolute value. Finally, major clades are sampled with similar proportions of species, avoiding among-clade biases in estimating speciation and/or extinction rates (see methods, Fig. 2, Table. S4). All these considerations suggest a methodologically robust association between heterostyly and decreased extinction rather than increased speciation. Hence, the floral biology of Primulaceae and our finding of a decrease in extinction jointly

suggests that the association of heterostyly and increased diversification is not convincingly explained by a high incidence of pollinator shifts.

Rather than providing a mechanism for higher diversification rates via pollinator shifts, heterostyly may instead convey genetic advantages associated with the obligate outcrossing that it enforces via heteromorphic self-incompatibility (Fig. 1). Population-genetic theory predicts that, compared to selfing, outcrossing reduces inbreeding, leading to larger effective population sizes, decreasing the fixation of slightly deleterious alleles (Wright et al. 2008), allowing for the maintenance of higher genetic diversity (Lloyd 1980, Hamrick & Godt 1998) and facilitating adaptation to changing environmental conditions. The mentioned genetic processes are jointly thought to promote long-term evolutionary survival by mitigating the risks of extinction (Takebayashi & Morell 2001; Frankham 2005; Escobar 2010). Comparable evidence for an association between outcrossing, high net diversification and low extinction was found in a family-wide analysis of Solanaceae, which detected lower rates of extinction and species turnover in self-incompatible than self-compatible taxa (Goldberg et al. 2010). Similarly, an angiosperm-wide analysis suggested that net diversification rates of families with mechanisms for self-sterility were higher than those of families without such mechanisms (Ferrer & Good 2012). Our results corroborate the idea that the population genetic advantages of outcrossing for long-term lineage survival may spur the proliferation of clades over millions of years, by demonstrating a robust phylogenetic relationship between decreased extinction rates and the origin of heterostyly in /Primula 35-15 Ma (node c in Fig. 2; Fig S1BC). The finding that heterostyly is associated with lower extinction is striking, because the floral syndrome could have been expected to drive higher extinction rates in the alpine/arctic habitats favored by primroses, where pollinator services needed for inter-morph pollen transfer are typically scarce or unreliable. Nevertheless our results suggest that, on macro-evolutionary timescales, the long-term genetic advantages of outcrossing outweigh risks associated with pollinator dependence.

Innovation and opportunity during the diversification of Primulaceae

In contrast to the paucity of convincing evidence that key morphological innovations have resulted in accelerated rates of species diversification, correlations between high rates of diversification and extrinsic opportunities afforded by shifts to particular biogeographic regions have been reported more commonly, for instance in the Andes (Hughes & Eastwood 2006, Moore & Donoghue 2007, Drummond et al 2012), the Mediterranean region (Valente et al. 2010), or the Hawaiian archipelago (Baldwin & Sanderson, 1998). It has even been suggested that geography is a more important predictor of species richness among angiosperm families than morphology or the amount of time available for diversification (Vamosi & Vamosi 2010, 2011), presumably because environmental conditions (e.g., the degree of habitat heterogeneity) may define the "carrying capacity" of a geographic region. The Eastern Himalayan region, which harbors about 80% of the extant diversity of Primulaceae, is physiographically one of the most heterogeneous regions worldwide, suggesting a high "carrying capacity" and ample opportunity for extrinsically driven, high diversification rates (Qiu et al. 2012). Indeed, several phylogenetic studies supported a rapid diversification linked to high physiographic diversity, mountain uplift, and climatic instability since the late Tertiary in the Eastern Himalaya (e.g. *Rheum*, Polygonaceae, Wang et al. 2005; *Gentiana*, Gentianaceae, Zhang et al. 2009; *Saussurea,* Asteraceae Wang et al. 2009).

Interactions between morphological innovation and ecological opportunity potentially offer powerful explanations for among-lineage differences in the accumulation of species over time (Wagner et al. 2012). For instance, evidence suggests that succulence promoted faster radiation after the onset of aridification of the

climate in the late Miocene (Klak et al. 2004, Arakaki et al. 2011) and the evolution of perennial habit in *Lupinus* facilitated subsequent accelerated diversification and range expansion into newly available montane habitats after mountain uplift in South America (Drummond et al. 2012). However, there is no obvious biological/physiological mechanism that might explain a higher ability of heterostylous lineages to exploit extreme physiographical heterogeneity as compared to their non-heterostylous progenitors. Furthermore, our results, showing that heterostyly primarily affects extinction rather than speciation rates (Fig. 3), do not corroborate a scenario of increased speciation via niche colonization in heterostylous primroses. Additionally, both the heterostylous and non-heterostylous clades of Primulaceae have the main center of species diversity in the Sino-Himalayan region and secondary centers in similar montane-alpine zones across the northern hemisphere (e.g., Hu & Kelso 1996, Smith & Lowe 1997, Richards 2003, Schneeweiss et al. 2004). In fact, species of *Primula* with heterostyly and *Androsace* with no heterostyly regularly co-occur and co-flower in these alpine habitats. In summary, the lack of any clear links between extrinsic factors and the higher diversification rate of /Primula underlines the importance of heterostyly in elucidating the pattern of differential diversification observed in the family.

Despite the likely lack of direct links between geological/ecological drivers and macro-evolutionary processes in Primulaceae, the extreme physiographic heterogeneity of the montane-alpine habitats typical of /Primula might interact with the higher adaptability afforded by the obligately outcrossing breeding system of heterostyly to drive higher net diversification rates. In heterogeneous habitats, outcrossed, heterostylous species are more likely to include at least some individuals with genetic combinations that are adaptive for a diversity of ecological niches or changes of ecological variables, while inbreeding, non-heterostylous species might more easily become extinct if the environment changes (Frankham 2005; Glémin & Ronfort 2012). Therefore, the innovation of heterostyly might have synergistically interacted with the ecological opportunities available in alpine habitats to reduce extinction rates in the heterostylous /Primula, thus increasing diversification rates in this clade (Figs. 2, 3)

Contrasting effects on long and short timescales

Intriguingly, the loss of heterostyly does not appear to have the inverse effect of its gain, i.e., non-heterostylous Primulaceae may have higher diversification rates than the heterostylous lineages. For instance, the Medusa analysis found two secondary increases in diversification rate deeply nested in the phylogeny (Fig 2; brown and purple clades), one of which in the /Primula clade and appearing to be associated with the loss of heterostyly in species of *Primula* section *Aleuritia*. In congruence, an additional BiSSE analysis that included all heterostylous and non-heterostylous species in /Primula indicated that, overall, the loss of heterostyly in this clade is associated with increased speciation and net diversification rates (Table S3), suggesting that the contrast between the effects of the loss of heterostyly in /Primula and the gain of heterostyly in Primulaceae represents a more general phenomenon then one that is tied exclusively to the specific intricacies of Section *Aleuritia*.

The loss of heterostyly in *Primula* is thought to be a Mendelian process caused by crossing-over in the heterostyly S-locus supergene, giving rise to mutants (often termed homostyles) that combine male and female aspects of both floral morphs, and sometimes (e.g. in Section *Aleuritia*) relates to polyploidization (Lewis & Jones 1992, Guggisberg et al. 2006, Barrett & Shore 2008, Cohen 2010, Naiki 2012). Such plants are generally thought to be highly self-fertile, based on experimental evidence (e.g. Scott 1865; Schaeppi 1935; Carlson et al. 2010; De Vos et al. 2012), though genetic selfing rates are rarely reported (Piper et al. 1984).

The higher speciation and diversification rates of species in Section *Aleuritia* deeply nested within /Primula can be understood in the context of polyploid speciation via secondary contact during Pleistocene cycles (Guggisberg et al. 2006, 2009). The climatic oscillations of the Pleistocene caused species ranges to repeatedly expand and contract, allowing populations to become genetically differentiated in isolation, then come into contact again and occasionally hybridize. When hybridization was stabilized by polyploidization, new species had an opportunity to evolve. The higher recombination rates triggered by polyploidization also promoted recombination at the heterostyly supergene, with the ensuing loss of heterostyly (Guggisberg et al. 2006, 2009). Hence, polyploid speciation via secondary contact provides a plausible mechanism for the rapid radiation that Medusa detected in Section *Aleuritia*.

At first sight, the finding of high diversification rates associated with secondary loss of heterostyly seems contradictory to the overall finding of high rates of diversification following the gain of heterostyly, but this is likely due to delay in the mechanisms through which heterostyly buffers against extinction compared to the mechanism through which the loss of heterostyly may spur speciation. The possible benefits of the loss of heterostyly and becoming self-compatible in terms of the rate of speciation are likely more or less instantaneous: when associated with the incidence of polyploidization via cytogenetic incompatibilities that enforce reproductive isolation; irrespective of polyploidy, self-compatibility immediately increases the likelihood of allopatric speciation, given that self-compatible lineages are more likely to found new populations following (long-distance) dispersal than self-incompatible lineages (Baker 1955). Furthermore, selfing decreases the effective population size, which should increase the efficacy of selecting recessive alleles of small beneficial effect from standing genetic variation (Wright et al. 2008), increasing the rate at which adaptation may occur (Glémin & Ronfort 2012). In contrast, the causes for extinction in self-compatible lineages relate to their depauperate genetic diversity and fixation of slightly deleterious mutations (Frankham 2005), which would take considerable time to accumulate, making extinction potentially a slower process compared to speciation. Thus, although non-heterostylous species may originate often and perhaps radiate faster than heterostylous species over a limited time period (the distribution of non-heterostylous tips in /Primula suggests during < ca. 3MY; Fig. 2), these lineages tend to "live fast and die young", as the short-term advantages of selfing are offset over longer timescales (>20 MY since the origin of heterostyly) by the genetic benefits of heterostyly and obligate outcrossing.

Results from other studies are congruent with this interpretation, suggesting that the time-scale dependent effect of a trait on diversification dynamics may be a general phenomenon. The negative effects of selfing were related to high extinction and low diversification rates in self-compatible species when studied over long time scales (family level) in Solanaceae (Goldberg et al. 2010), whereas lack of outcrossing is related to increased diversification rates over shorter timescales (species level) within *Oenanthera* (Johnson et al. 2011). Similarly, polyploid lineages were shown to originate readily, yet not persist into deep evolutionary time (Mayrose et al. 2011). The interpretation of our results that traits may have contrasting effects on long and short time scales suggests that macro-evolutionary patterns observed over a short timescales (e.g., rapid bursts of speciation in a species flock; fast polyploid speciation upon secondary contact during Pleistocene glacial cycles) may not necessarily translate into phenomena that are important over timescales that are orders of magnitude longer, with potentially important implications for our understanding of the diversification of life as a whole.

Heterostyly and the homoplasious history of angiosperm diversification

Not only is the heterostylous /Primula clade more species rich than its non heterostylous relatives /Soldanella and /Androsace, but our analyses also established that more heterostylous species exist than expected from background diversification rates in non-heterostylous taxa (Fig. 4). However, the higher species diversity associated with heterostyly in Primulaceae is not evident in all the 28 families that include heterostylous species (Naiki 2012), which may have several reasons. The term heterostyly belies considerable complexity in floral shape, ecology, habitat, etc. (Barrett 1992), thus diversification dynamics driven by other factors than heterostyly may be more important in other groups. It is also possible that the evolutionary fates of heterostylous and related non-heterostylous lineages strongly depend on ecological and physiographic settings of clades. Moreover, the main effect of heterostyly, a low rate of extinction over long macro-evolutionary time scales, would only result in different numbers of species among clades if extinction in related, non-heterostylous species is higher (Klak et al. 2004). However, in most groups, the phylogenetic relationships of heterostylous species, the age of the origin of heterostyly, or the floral biology of related non-heterostylous species remain largely unknown. Indeed, known phylogenetic patterns of heterostyly appear to be quite different among families. For instance, in Rubiaceae, as in Primulaceae, heterostyly evolved early, and was lost repeatedly (Ferrero et al. 2012), but in *Nymphoides* (Menyanthaceae), heterostyly was gained, lost, and regained (Tippery & Les 2011), while in the tribe Lithospermae (Boraginaceae), at least ten independent origins occurred, with no apparent losses (Cohen 2012). These examples illustrate the complexity of possible diversification patterns across heterostylous angiosperms. Detailed phylogenetic and diversification rate analyses with broad sampling, including related, non-heterostylous groups, are clearly needed to evaluate the macro-evolutionary effects of heterostyly on a clade-by-clade basis.

Heterostyly is one of the many complex, yet convergent floral innovations that characterize angiosperms and its phylogenetic history, as elucidated for Primulaceae, apparently exemplifies the often complex diversification patterns observed in angiosperms. The convergent occurrence of heterostyly across 28 plant families provides excellent opportunities to investigate the impacts of complex floral traits on species diversification over a range of evolutionary time scales (Cohen 2010; Wake et al. 2011). The apparent plasticity of angiosperms (Crepet & Niklas 2009) suggests that even complex floral traits may be relatively easy to evolve (Cohen 2010). Our results demonstrate that floral traits that affect plant mating may have dramatic effects on macro-evolutionary patterns of species diversification, epitomized by the 20x imbalance in species numbers between *Primula* s.l. and its sister group, the *Soldanella* clade. While impacts of plant reproductive systems on diversification dynamics have been widely predicted and are suggested to have been key drivers of diversification in flowering plants, they have rarely been documented so far. The complex evolutionary fates of heterostylous lineages, where gains and losses may have contrasting effects on short and long evolutionary timescales, fits well with the overall plasticity and dynamic diversification history that may ultimately account for the extraordinary success of angiosperms.

Methods

The analytical pipeline to test the effects of heterostyly on diversification is illustrated in the Appendix, Fig Sx, and details are described in the Appendix, Text S1.

Taxon sampling and phylogeny inference

We sampled all 11 genera of Primulaceae s.str., including all sections of the two large genera Primula and Androsace (38 and 6 sections, respectively; Table S4), aiming for including species numbers proportional to the size of genera and sections (Tables S4, S5), and proportional to the occurence of heterostyly (present in 62% and 60% of extant and sampled species, respectively). This sampling strategy minimized the risk of artifactual results due to biased or incomplete taxon sampling.

Phylogenetic relationships among the 265 sampled species were based on coding (*mat*K) and non-coding (*trn*L, *trn*L-*trn*F, *rpl*16) chloroplast loci, which were generated de novo (210 sequences) or downloaded from genbank (820 sequences). Alignments were obtained using Muscle (Edgar, 2004) with manual adjustments and contained 5.9% gaps or missing data. After preliminary runs with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), we determined that the sequence data was best concatenated and partitioned by region and codon position based on BayesFactors (Posada & Buckley 2004) calculated with Tracer v1.5 (Rambaut & Drummond 2007). The substitution model GTR+G was employed for each partition as selected after calculating AIC scores with ModelTest (Posada & Crandall 1998).

Divergence times and phylogenetic relationships were jointly estimated using BEAST 1.6.2 (Drummond & Rambaut 2007), applying Bayesian relaxed clocks with uncorrelated lognormal (UCLN) and uncorrelated exponential (UCEXP) distributions of rate variation among branches. We generated a posterior distribution of chronograms and a maximum clade credibility (MCC) tree based on each dating method seperately and performed all diversification-rate analyses on both sets of results, because we noted that the tree shape of chronograms may be affected by the dating method used, which we suspect may bias diversification rate analyses. Chronograms were calibrated with prior distributions based on fossil seeds of *Primula riosiae* from the Miocene (Czaja 2003) and based on results from a separate analysis of a six-locus, 21-taxon chloroplast DNA sequence of Ericales (Table S6), that was itself calibrated with the fossil taxa *Eurya* and *Saurauia* from the Upper Cretaceous (Knobloch & Mai 1986).

Character history reconstructions

Presence of heterostyly was scored based on floristic treatments, taxonomic literature, and Ernst's (1962) extensive discussion of breeding systems in *Primula* (Table). We employed three character coding schemes devised to rigorously assess wether the state of ten taxa for which both heterostyly and no heterostyly was reported affected the character reconstructions (Table S5). Additionally, we assessed the effect of the scoring of atypical heterostyly in *Androsace vitaliana* and *Hottonia palustris* in a fourth coding scheme (Table S5). Character history was reconstructed using BayesTraits v.1.0 (Bayesian and Maximum Likelihood inference; Pagel & Meade 2007) and Mesquite (Parsimony; Maddison and Maddison 2011) and statistical support for heterostyly as ancestral state was evaluated for five key internal nodes under each coding scheme based on BayesFactors, for which marginal-likelihoods were obtained using the harmonic-mean estimator implemented in BayesTraits.

Diversification rate analyses

Diversification rate shifts were detected using Medusa (Alfaro et al. 2009), implemented in the geiger package of the R statistical envrionment (R Development Core Team, http://www.r-project.org, version 2.14.2), executed on MCC trees and on each of 100 trees from the posterior distribution of trees, and corroborated using SymmeTree v.1.1 (Chan & Moore 2004). Medusa uses maximum likelihood to find a birth-death model of

diversification with the optimal number and position of rate shifts, by fitting increasingly complex models, while penalizing for excess parameters based on AICc-scores. We did not employ a correction for incomplete taxon sampling, because it was not possible to assign unsampled taxa to extant clades, moreover, we deliberately devised taxon sampling to already proportionally represent taxonomic and breeding-system diversity. Medusa has the disadvantage that AICc scores can be biased toward favoring complex models (i.e. with more rate shifts) when the amount of data decreases. In contrast, SymmeTree uses stochastic simulations to determine the correct distribution of the test statistic for every branch of the tree (Moore et al. 2004), thereby circumventing the need for an arbitrary ΔAICc cut-off.

We used BayesRate v.1.3.41 (Silvestro et al. 2011) to determine the diversification model that best describes the difference in speciation and extinction rates (if any) between the heterostylous clade /Primula, which contains >99% of all heterostylous species, and the non-heterostylous paraphyletic grade comprising /Soldanella + /Androsace. The analysis uses a posterior distribution of trees, each of which is split in two partitions: /Primula, and the rest. A birth-death model is then fitted that has a partition-specific parameter for both speciation and extinction rates (four parameters in total). In two subsequent runs, simpler models are fitted in which either the speciation or extinction rate is constraint to be equal between the partitions, and in a final run, a simple model without identifying tree partitions is fitted. Among these four models, the best model was selected using BayesFactors based on marginal likelihoods that were estimated via thermodynamic integration, which is known to perform robustly (Silvestro et al. 2011). This procedure allows to determine if shifts in speciation rate, extinction rate, or both account for the shift in net diversification rate inferred by Medusa and Symmetree, while fully accounting for uncertainty in tree reconstruction, divergence time estimation, and speciation- and extinction-rate estimation.

We also estimated character-state associated speciation and extinction rates using BiSSE (Maddison et al. 2007; FitzJohn et al. 2009), implemented in the R-package Diversitree v.0.9-3 (FitzJohn 2012). BiSSE fits a six-parameter model on a posterior distribution of trees, jointly estimating speciation and extinction rates associated with heterostyly and non-heterostyly, and rates of transition from non-heterostyly to heterostyly and thus accounting for uncertainty in the position in the tree where heterostyly evolved. However, in contrast to BayesRate, BiSSE does not allow for robust Bayesian model selection based on thermodynamic integration.

Finally, we asked the question "*How many species would we expect the heterostylous clade to contain, if heterostyly did not spur diversification rates?*" and answered this using Posterior Predictive Diversity Densities (Moore & Donoghue 2009). This approach exploits the posterior probabilities of the timing of the evolution of heterostyly, *t*, and the background diversification rate in the non-heterostylous tree partition, *r*, to generate a predictive probability distribution of species, while fully accounting for uncertainties associated with estimating *t* and *r*. The realized species diversity (i.e. the number of heterostylous species in /Primula) is then compared to this predictive diversity distribution to determine if rates of species diversification increased significantly since heterostyly evolved. We also determined the effect on the results of using birth-death vs. pure-birth models of diversification and a correction for missing taxa while calculating *r*.

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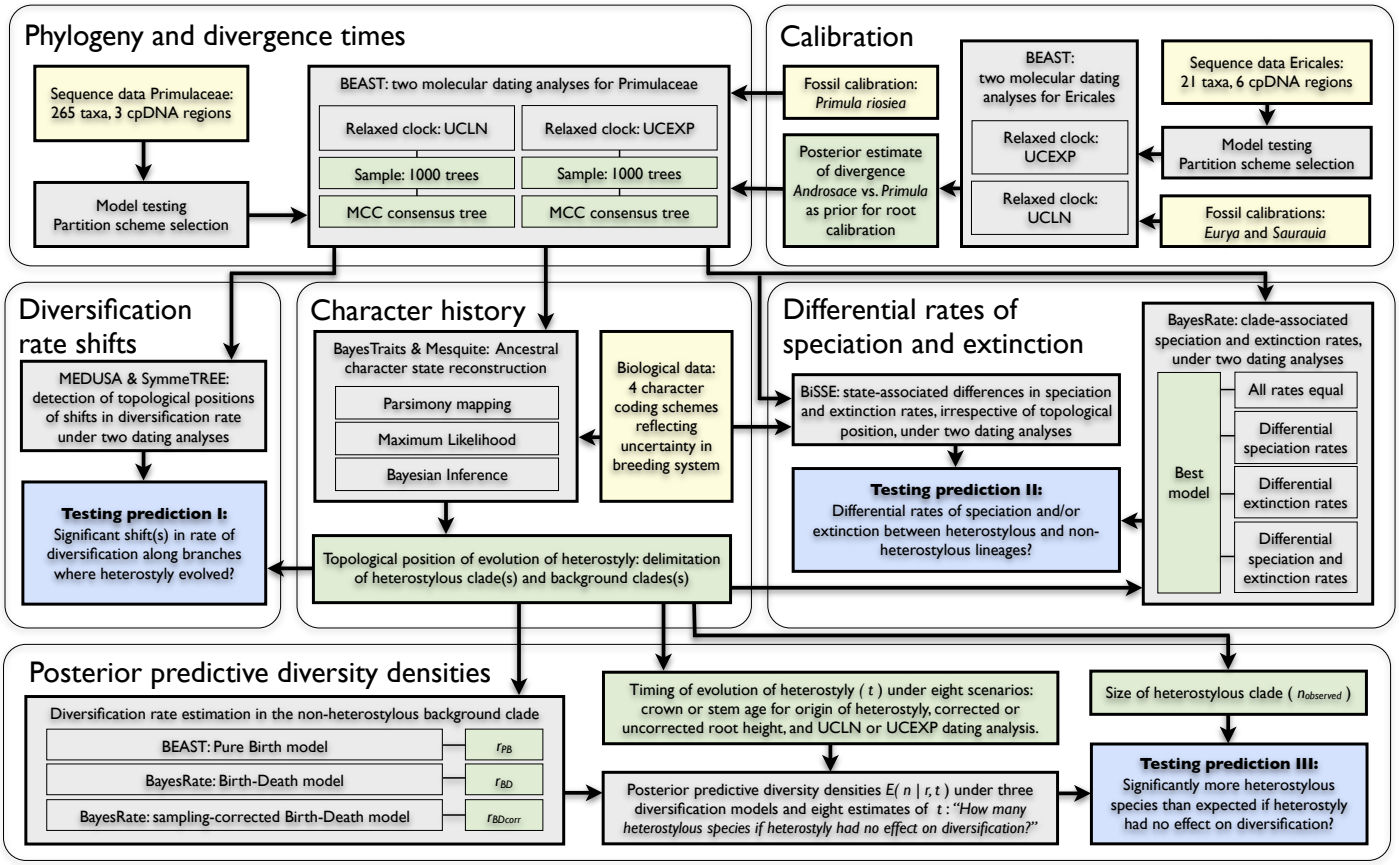
Appendix

Supplementary Information belonging to Chapter 2, containing Text S1, Figs S1-S4; Table S1-S5.

Text S1

Details of the analytical pipeline to evaluate the effects of heterostyly on diversification in Primulaceae is illustrated in the flowchart below, and details are described in the following text.



Flowchart illustrating the analytical pipeline employed in this study. The data used in this study (yellow) was used in analyses (grey) that yielded intermediate results (green), enabling to test the three predictions (blue) associated with the hypothesis that heterostyly promoted the diversification of primroses.

Sampling strategy

By sampling all genera and sections proportionally to the total number of species and to the number of heterostylous and non-heterosylous species (Table S4, S5), we accounted for missing species as good as possible given the current knowledge of the phylogeny of Primulaceae. Traditional taxonomic delimination of groups below the genus level (in subgenera and sections) based on morphological characters often does not reflect phylogenetic affinities of species (e.g. Schneeweiss et al. 2004; Mast et al. 2006; Yan et al. 2009). Hence, by including species from all genera and sections, we represent the morphological diverstity well, although we cannot assign unsampled species to any specific tips in downstream diversification analyses. Moreover, we avoid artifacts stemming from sampling one clade more intensely than others: the fraction of extant species that are sampled is similar for heterostylous and non-heterostylous species, as well as for the

three main clades in Primulaceae (Table S4). For *Primula,* we generally sampled one accession per species if it was known to be monomorphic for breeding system and two accessions if it included both heterostylous and a non-heterostylous subspecies (e.g., *Primula cuneifolia*). Information on species numbers and breeding systems was drawn primarily from Richards (2003), Hu & Kelso (1996), and Ernst (1962) (see Table S5 for details). In total, our dataset included 265 taxa, representing 36-38% of extant diversity (the exact percentage depends on the taxonomic authority followed, see Note 2 in Table S4), the most complete sampling of Primulaceae in a phylogenetic study to date.

DNA amplification, sequencing, and sequence alignment

Total genomic DNA was extracted from silica-dried leaves as described in Mast et al. (2006) and Schneeweiss et al. (2004). We amplified and sequenced loci of the chloroplast genome that allowed us to combine our newly generated data with existing sequence data for /Primula (Mast et al., 2006; Yan et al. 2009), /Androsace (Schneeweiss et al. 2004) and /Soldanella (Mast et al. 2006). DNA sequences from the tRNA-Leu (*trn*L) intron and the *trn*L-*trn*F intergenic spacer (hereafter trnLF), and the maturase K gene (hereafter matK) were obtained for all taxa, those from the intron of the ribosomal protein L16 (hereafter rpl16) for 89% of taxa. A total of 210 new DNA sequences were generated for 73 species as part of this study and then included in a matrix with 820 additional sequences retrieved from GenBank (Table S4). PCR amplification followed Mast et al. (2004), but with 32 cycles (or up to 36 if yields were low) of 30 s at 95º, 60 s at 52º, and 100 s at 72º, followed by a final extension period of 10 min at 72º, using the following primers. TrnLF: trnc, trnd, trne, trnf (Taberlet et al. 1991); matK: MatK1F, MatK3F, MatK1R (Sang et al. 1997), and the newly designed primers MatK3Frc (5'-ATG CAA AGA AGA GGC ATC TT-3', i.e., the reverse complement of primer MatK3F), MatK4F (5'-TTT CTT GTG CTA GAA CTT TGG-3', which anneals between MatK3F and MatK1R); rpl16: F71 (Jordan et al. 1996), R1516 (Baum et al. 1998). PCR products were cleaned using Qiaquick spin columns (Qiagen AG, l.c.) and cycle-sequencing was performed using BigDye Terminator v.3.1 (Applied Biosystems, Foster City CA, USA), using the manufacturer's protocols. Sequencing products were purified with Sephadex G-50 fine grade (GE Healthcare, Glattbrugg/Zürich, Switzerland), and loaded on a 3130xl DNA Analyzer (Applied Biosystems, l.c.). Electropherograms were checked and assembled into consensus sequences using the Staden Package v.1.6.0 (http://staden.sourceforge.net/). Alignments for each region were obtained using MUSCLE (Edgar, 2004) with manual corrections, and sites with >50% missing data were excluded from the analyses; this procedure also removed all ambiguously aligned hyper-variable regions. Our final dataset contained 3351 sites for 265 taxa and consisted of 94.1% non-missing data.

Phylogeny estimation

Primulaceae phylogeny and divergence times were jointly estimated in a Bayesian context using BEAST v.1.6.2 (Drummond & Rambaut 2007). Prior to estimation, we determined an optimal sequence partitioning scheme separating regions and codon positions (5 partitions), by comparing four competing schemes with BayesFactors (Posada & Buckley 2004) using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003), employing 2 MCMC runs of 15 million generations each with default settings. ΔAIC tests (Posada and Buckley, 2004) using Modeltest v.3.7 (Posada & Crandall 1998) suggested GTR+G as the most suitable model of sequence evolution for all partitions, when selecting the most parameter-rich model within 2 AIC units of

the best model to avoid under-parameterization (Lemmon and Moriarty, 2004). We avoided models that incorporated both parameters I (i.e., proportion of invariable sites) and Γ (i.e., gamma-distributed rate variation among sites), because several initial runs showed considerable parameter interaction. Unless stated otherwise, marginal likelihoods for all analyses employing BayesFactors were calculated using the harmonic mean estimator implemented in Tracer v.1.5 (Drummond & Rambaut, 2007), using post-burnin samples from an MCMC of which performance and convergence of independent runs were assessed using Tracer and statistics provided by MrBayes v.3.1.2.

Divergence time estimation

Initial BEAST (Drummond & Rambaut 2007) runs suggested that the use of different models of variation in substitution rates among branches (i.e., "relaxed clock" models) might affect branching times in the phylogeny, with possible downstream effects on analyses of diversification rates. Therefore, we performed two BEAST dating analyses, using the uncorrelated lognormal relaxed (UCLN) and uncorrelated exponential relaxed (UCEXP) models, to explore the sensitivity of the inferred divergence time estimates to different assumptions. Dating analyses employed a birth-death branching process and default priors in BEAST (Drummond & Rambaut 2007). The three main clades /Primula, /Soldanella, /Androsace, which are strongly supported by the MrBayes analyses with optimal partitioning scheme (posterior probability 1.0; Fig. S1A), were constrained to be monophyletic. Both dating analyses employed six parallel runs of 50 million generations, sampling every 1,000th generation (trees every 2500th to reduce file size), which were combined after discarding 10% burnin and assessing MCMC performance and convergence using Tracer and AWTY (Wilgenbusch et al. 2004). The resulting two posterior distributions of phylogeny estimates were each downsampled to 10000 trees and summarized as maximum clade credibility (MCC) trees with median node heights. Subsequent analyses were performed based on the results of the UCLN analysis and on that of the UCEXP analysis.

Calibration

The fossil record of Primulaceae is inadequate to provide multiple, reliable calibrations within Primulaceae (see also Schneeweiss et al. 2004, Yesson et al. 2009, Boucher et al. 2012). Miocene seeds assigned to *Androsace* (Dorofeev, 1963, Łańcucka-Środoniowa 1966, 1979) cannot serve as calibration constraints, because they lack clear diagnostic morphological apomorphies with extant taxa. However, fossils seeds of *Primula riosiae*, also from the Miocene (Czaja 2003), can be used to assign a minimum age of 15.97 Ma (the early-mid Miocene boundary) to the split between /Primula and /Soldanella. Alongside the direct fossil calibration a secondary calibration strategy to generate a second time-constraint for the root node was also adopted. Criticism of this strategy (Ho 2007) was addressed by calculating a prior probability distribution for the root that fully incorporated the posterior distribution of the age of this calibration point. To this end, DNA sequences of three coding (*mat*K, *ndh*F, *rbc*L) and three non-coding chloroplast regions (*trn*L, *rps*16, *trn*V) were downloaded from GenBank to assemble a matrix of 8446 aligned sites and 21 taxa for Ericales (which include Primulaceae), with 4.7% missing data (Table S6). Sequence alignment and subsequent analyses followed the protocols described above for Primulaceae, unless otherwise stated. Fossils of *Eurya* (Santonian) and *Saurauia* (Turonian; Knobloch & Mai 1986) were applied as lognormal priors to constrain the divergence

between Actinidiaceae and Roridulaceae and between *Pentaphylax* and *Ternstroemia* (Pentaphylacaceae), respectively (see also Bremer et al. 2004). A maximum age of 125 Ma (Barremian-Aptian boundary) was assigned to the root, corresponding to the oldest pollen record for Eudictos (Magallon and Sanderson 2001, Doyle & Hotton 1991). Stratigraphic age estimates followed the International Commission of Stratigraphy, September 2010 (http://www.stratigraphy.org). The age of the Primula-Androsace split from these different clock models was estimated in two separate BEAST analyses of six runs each that differed in the use of the UCEXP or UCLN relaxed clock models. BayesFactors indicated that the UCLN and UCEXP clocks performed equally well: the marginal likelihoods of the analyses were -42055.041 +/- 0.223 and -42055.195 +/- 0.238, respectively. However inferred ages of the *Primula* - *Androsace* split differed: UCLN, mean age 41.8829 MY, 95% highest posterior density (HPD) 24.226 - 60.3421 MY; UCEXP, mean age 36.371 MY, 95% HPD 18.7883 - 55.0718 MY. Therefore, we created a calibration prior for the root of the Primulaceae dataset incorporating the age estimates for the *Primula-Androsace* split from both clock models. To this end, we constructed a joint marginal distribution by adding equal numbers of samples from the posterior distributions of both calibration analyses. This joint-marginal distribution had its point of highest posterior density at 38.82 MY, 95% HPD 21.0891 - 58.9038 MY. As a calibration prior, we approximated the joint-marginal distribution with a Normal distribution mean 39.99645 and standard deviation 11.492, which has identical 95% HPD interval.

Character history

Heterostyly occurs in ca. 85% of all species in /Primula (Table S4). In this clade, stigmas and anthers are embedded typically in the middle of the corolla tubes of short-styled and long-styled morphs, respectively, whereas the alternate sexual organs are placed at the mouth of the corolla tubes in the respective morphs (Fig. 1). *Hottonia palustris* (/Soldanella) and *Androsace vitaliana* (/Androsace) are also distylous, but in a form that deviates from that typical of Primula. The short-style morph of *Hottonia palustris* has long filaments that are largely free, with the anthers exerted above the flowers (Schaeppi, 1934), while *Androsace vitaliana* has strong stylar, but weak staminal dimorphism (Schaeppi 1935; pers. obs.). Most heterostylous species appear to be self-incompatible (Richards 2003), while non-heterostylous species commonly produce seeds after self-fertilization (see, e.g., Schaeppi 1934; Richards 2003; Huang et al. 2006; De Vos et al. 2012). Because the latter species have flowers of a variety of shapes, sizes and breeding systems, we refrain from using the term "homostylous", which typically refers to florally monomorphic species secondarily derived from heterostylous ancestors (see Mast et al. 2006). Presence of heterostyly was scored as a binary trait, in four different character-coding schemes. Three schemes were designed to appropriately deal with ten species that have been claimed to be heterostylous in some, but not all populations, or about which the available data are inconclusive: scheme 1: these species were scored as heterostylous; scheme 2: scored according to predominant breeding system for the species, based on Ernst (1962) and taxonomic descriptions; scheme 3: scored as non-heterostylous; scheme 4: *Androsace vitaliana* and *Hottonia palustris* were scored as non-heterostylous, because of their atypical floral polymorphism, whilst employing scheme 2 for all other species (see Table S5). Comparing the results obtained using the four character coding schemes allowed for a more rigorous assessment of the effect of polymorphic species on character reconstructions than possible when employing a character coding scheme that includes multi-state scoring.

Based on the four character coding schemes, we determined the ancestral states at five key internal nodes (a-e, Fig. 2), using Bayesian and Maximum Likelihood inference implemented in BayesTraits v.1.0 (Pagel & Meade 2007) and parsimony mapping in Mesquite (Maddison & Maddison 2011). All analyses were performed on a sample of 1000 trees from the posterior distribution under both dating analyses. Parsimony mappings used equal weighting of gains and losses, because a previous study showed no qualitative effect of different weighting schemes on the origin of heterostyly in /Primula (Mast et al. 2006). Bayesian (BI) and Maximum Likelihood (ML) analyses employed a model with separate parameters for forward and reverse rates (i.e. MK2), which fitted the data better than a symmetrical model in initial runs. ML analyses calculated the proportion of likelihood associated with selected backbone nodes being heterostylous or not, averaged across 1000 trees from the posterior distributions of trees to reflect phylogenetic uncertainty. BI analyses inferred the 95% highest posterior density interval for the five nodes of interest to be heterostylous, while averaging across trees during MCMC. Statistical support for heterostyly as the ancestral state was determined by constraining each of the five nodes of interest in turn to be either heterostylous or non-heterostylous and performing an MCMC analysis. This way, we inferred the marginal likelihood associated with each state at nodes of interest (using the harmonic mean estimator implemented in BayesTraits) and calculated the BayesFactor to infer if either state was significantly supported. MCMC chains were sampled every 1000th generation for 10 million generations, after a burnin phase of 500,000 generations. To achieve adequate MCMC performance, we adjusted the proposal window by setting the rate deviation to 0.05 and employed exponential priors for rate parameters with mean 1, after an initial set of runs. We obtained qualitatively identical results using uniform priors or exponential hyper-priors.

Detection of diversification rate shifts

We used the function MEDUSA from the geiger 1.3-1 package (Alfaro et al. 2009) in R v.1.14.2 to test whether species diversification rates changed along branches where character reconstructions indicated that heterostyly evolved. Medusa analyses were executed on MCC trees from each of the two dating analyses, using a conservative AICc cut-off of 4. To determine if the number and positions of rate shifts on the MCC tree ware affected by uncertainty in reconstruction of phylogeny and branching times, we also ran a Medusa analysis on each of the 100 samples from the posterior distribution of trees, and checked if shifts associated with the evolution of heterostyly found in the MCC trees were also recovered from each sample of the posterior distribution. To corroborate the Medusa results, rate shifts were also detected using SymmeTree v.1.1 (Chan & Moore, 2005). We based the SymmeTree analysis on the 50% majority rule consensus trees from both dating analyses, rather than the MCC tree, in order to exploit the option of stochastically resolving unresolved nodes, thereby accounting for topological uncertainty.

Clade-associated speciation and extinction rates

A Bayesian diversification-rate analysis using BayesRate v.1.3.41 (Silvestro et al. 2011) was used to test for differential speciation and/or extinction rates between heterostylous and non-heterostylous tree partitions. The best model was selected among the four competing diversification models using BayesFactors, for which marginal likelihoods were obtained via thermodynamic integration, employing six scaling classes and exponential priors on speciation and extinction rate parameters. BayesRate analyses were performed using

100 trees from the posterior distribution of chronograms from each of the two dating analyses, with 10,000 post-burnin MCMC generations per tree, sampling every 10th generation. The differences in rates between the heterostylous and non-heterostylous tree partitions was visualized by plotting the differences in speciation, extinction, and net-diversification at every sampled generation of the MCMC. Congruent results were obtained when repeating the analyses after excluding all of the ca. 15% non-heterostylous species in /Primula.

State-associated speciation and extinction rates

We also estimated character-state associated speciation and extinction rates using BiSSE (Maddison et al. 2007), implemented in the R package Diversitree v. 0.9-3 (FitzJohn et al. 2012). We estimated the parameter values of an unconstrained BiSSE model based on character-coding scheme 2 across a sample of 1000 trees from the posterior distribution of trees, using slice sampling. Rate parameters received a relatively wide exponential prior with the mean specified as 2x the log of the number of taxa divided by the root age (ca. 0.29) as indicated by Johnson et al. (2011), although other prior settings did not qualitatively affect the results. The window size for slice sampling was optimized as recommended in the manual of the Diversitree package. To test if heterostylous lineages have different speciation, extinction and net diversification rates (i.e. speciation minus extinction), we plotted the distributions of difference between non-heterostyly and heterostyly-associated rates, and determined if the 95% interval of highest posterior density included 0 (Johnson et al. 2011). Hypothesis testing based on ΔAIC scores after maximum likelihood runs yielded qualitatively identical results.

Posterior predictive diversity densities

To address the question "*How many species would we expect the heterostylous clade to contain, if heterostyly did not spur diversification rates?*", we followed the approach of Moore & Donoghue (2009) using Posterior Predictive Diversity Densities. This approach exploits the posterior probability of the timing of the evolution of heterostyly, t, and the posterior probability for the diversification rate in the non-heterostylous tree partition, r, to generate the predictive probability distribution of species, $E(n \mid t, r)$, while taking into account uncertainties associated with estimating t and r. The realized species diversity is then compared to this predictive diversity distribution to determine if rates of species diversification increased significantly since heterostyly evolved.

In total, we calculated 24 distributions of $E(n \mid t, r)$, to fully capture uncertainty associated stemming from using different methods to estimate t and r. The time since heterostyly evolved, t, depends on the topological position of the evolution of heterostyly (the oldest node inferred to be heterostylous), and on the age of that node. We considered two topological scenarios: the most parsimonious character state reconstruction that used the crown node age of /Primula as the origin of heterostyly (node c in Fig. 2; "crown age", t_{crown}), or the alternative scenario whereby heterostyly evolved at the stem node of /Primula, a scenario not rejected by Maximum Likelihood and Bayesian analyses (node b in Fig. 2; "stem age", t_{stem}). The posterior distributions of age estimates of these two nodes were exracted from the results of the dating analyses described above. Confidence intervals of t were comparatively large; to investigate if this significantly impacted on the results, we performed two additional dating analyses in which the root height was corrected. Both were identical to the BEAST analyses described above, with the exception that the normal distribution at

the root was substituted for the point estimate of highest posterior probability of the *Androsace-Primula* split in the Ericales analyses, and all proposals that operate on the treeModel.rootHeight parameter were disabled. Hence, we obtained in total eight estimates of t , namely all combinations of using t_{crown} or t_{stem}, corrected or uncorrected root height, and UCLN or UCEXP dating analysis.

We used three different ways to estimate the background diversification rate, r, i.e. the rate at which species of the paraphyletic grade of /Soldanella plus /Androsace evolve. First, r was extracted from separate BEAST analyses that excluded the /Primula clade, following Moore & Donoghue (2009), with settings as described for the Primulaceae dataset, except that we used a Yule prior for the branching process (i.e. pure birth), ran the six runs for 13,333,000 generations, and excluded 25% as burnin to obtain 10,000 samples. To be able to correctly calculate $E(n \mid t, r)$, we performed these additional BEAST analyses four times, i.e., using a corrected or uncorrected root height, and UCLN or UCEXP molecular-clock model. The posterior predictive diversity density was calculated using Equation 3 of Moore & Donoghue (2009) and montecarlo integration executed in R v.1.5.0, by combining each posterior distribution of r under a dating scenario with the posterior distributions of t_{crown} and t_{stem} from the same calibration scenario. If, for instance, t was calculated based on UCLN analysis with corrected root height, we would also use the estimate of r based on a UCLN analysis with corrected root height

Secondly, as an alternative to the pure birth approach used by Moore & Donoghue (2009), we estimated the net diversification rate of the background, r, without assuming zero extinction, using BayesRate v.1.3.4 (Silvestro et al. 2011). Based on the results of the UCLN and UCEXP analysis, we fed the program 100 trees from the posterior distribution and used the implemented MCMC algorithm on each tree in turn to sample the posterior distribution of background diversification rates. At every generation of the MCMC, the expected number of species in the heterostylous clade, E, is calculated using $E = e^{(r*t)}$, where r is the background diversification rate currently sampled by the MCMC, and t is either the stem or the crown age for the evolution of heterostyly of the tree currently sampled. BayesRate analyses were run for 100,000 generations per tree, with 10% burnin, sampling every 100th generation, and using exponential priors on rate parameters. The resulting distribution of sampled values for E can be interpreted as the predictive diversity density calculated as described by Moore & Donoghue (2009).

Thirdly, we repeated the procedure with BayesRate, but used a correction for incomplete taxon sampling, by setting the sampling fraction of the background clade to 0.412 (see table SI_sampling for details on the extant and sampled number of species in the heterostylous and non-heterostylous tree partitions).

For each of the 24 posterior predictive densities (i.e. using all combinations of 2 age estimates for the origin of heterostyly, 2 root calibrations, 2 clock models, 3 models for background diversification) we tested if there are more extant heterostylous species than expected from the background diversification rates in non-heterostylous clades, by determining the proportion of predictive diversities that exceeds the realized diversity, using equation (4) in Moore & Donoghue (2009). To be conservative, we used the estimated number of heterostylous species in /Primula (n=457; see Table S4) as the realized diversity, rather than the full size of the clade.

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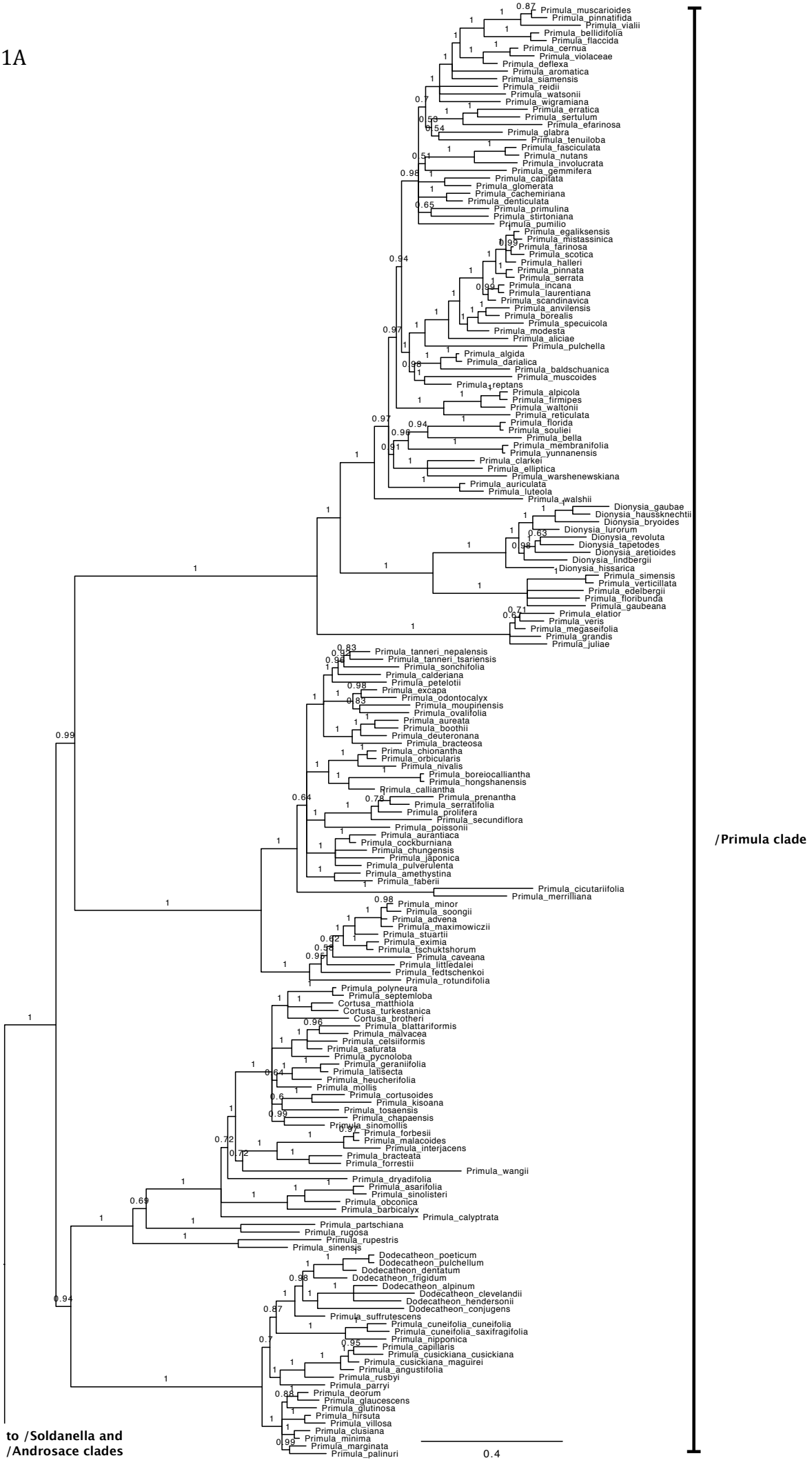
Fig. S1A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to /Soldanella and
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Fig. S1A (cont.)

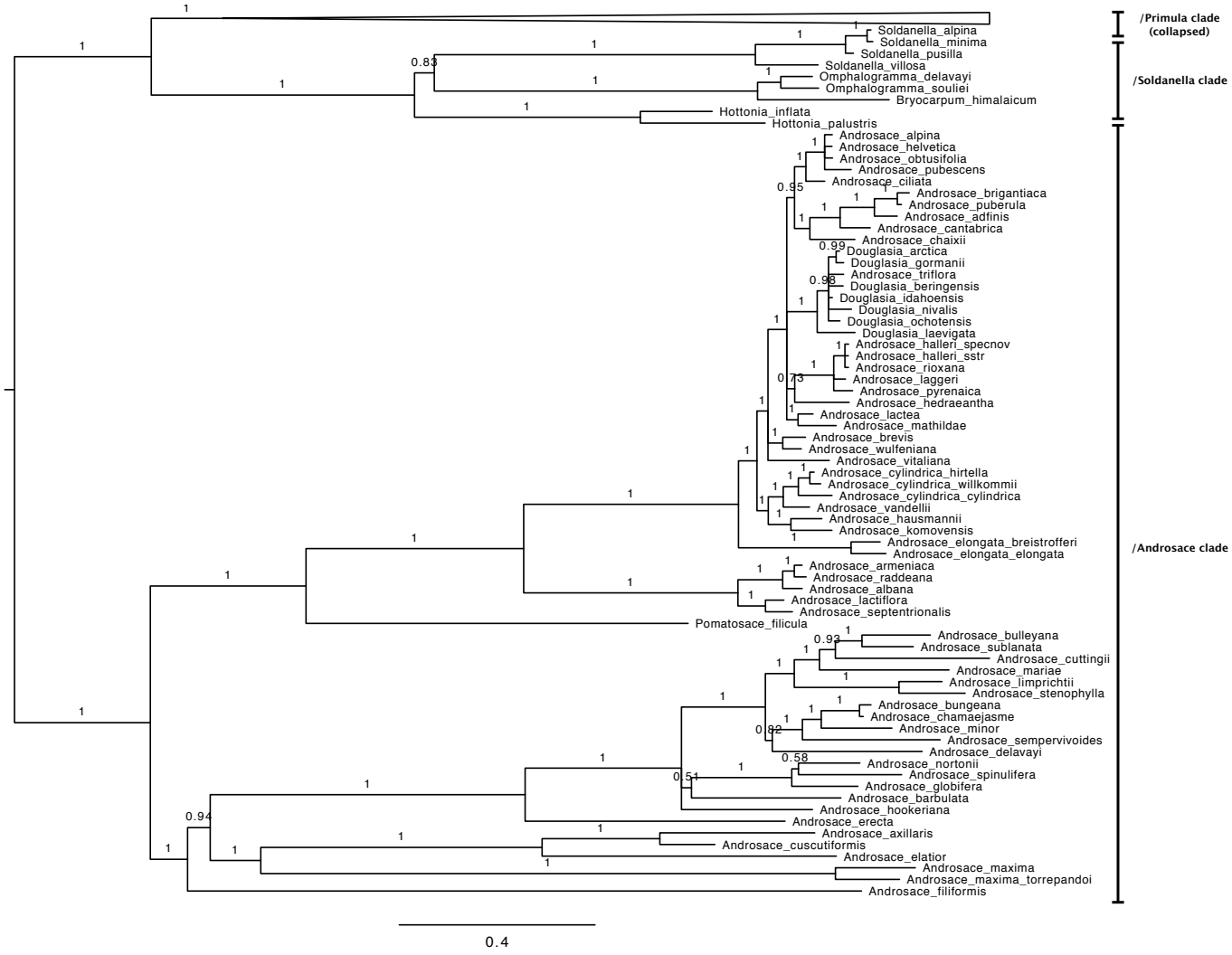
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Fig. S1B

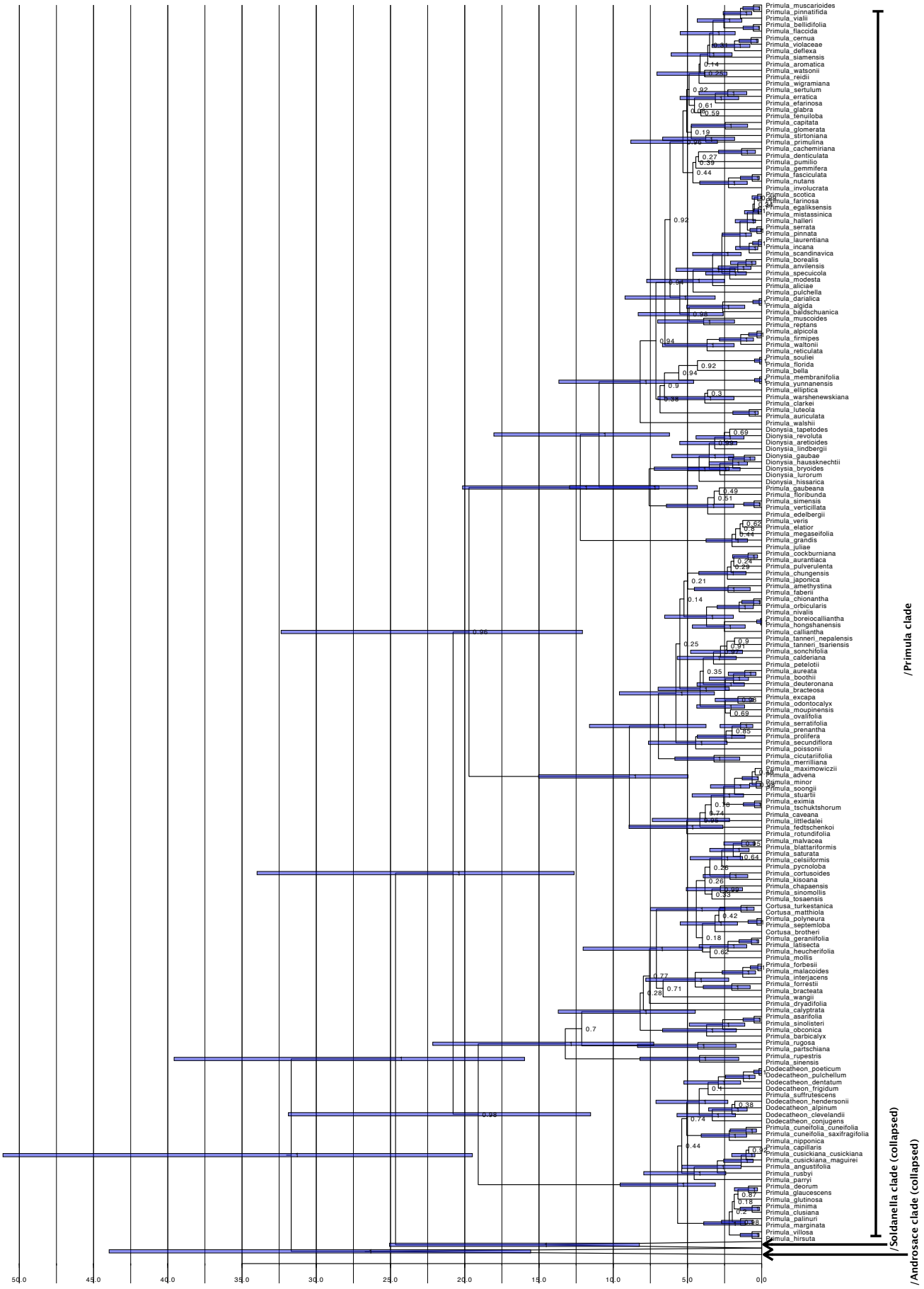
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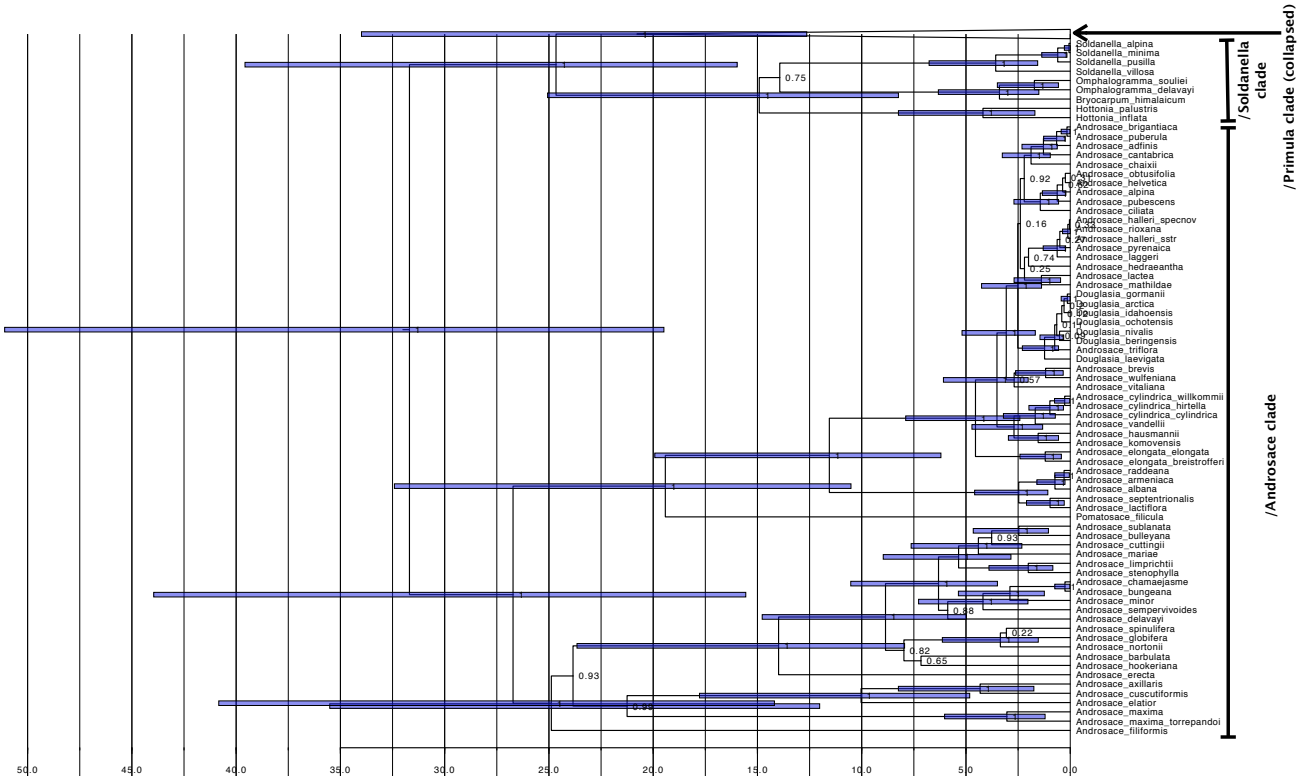
Fig. S1B (cont.)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Androsace_hedraeantha
Androsace_lactea
Androsace_mathildae
Douglasia_gormanii
Douglasia_arctica
Douglasia_idahoensis
Douglasia_ochotensis
Douglasia_nivalis
Douglasia_beringensis
Androsace_triflora
Douglasia_laevigata
Androsace_brevis
Androsace_wulfeniana
Androsace_vitaliana
Androsace_cylindrica_willkommii
Androsace_cylindrica_hirtella
Androsace_cylindrica_cylindrica
Androsace_vandellii
Androsace_hausmannii
Androsace_komovensis
Androsace_elongata_elongata
Androsace_elongata_breistrofferi
Androsace_raddeana
Androsace_armeniaca
Androsace_albana
Androsace_septentrionalis
Androsace_lactiflora
Pomatosace_filicula
Androsace_sublanata
Androsace_bulleyana
Androsace_cuttingii
Androsace_mariae
Androsace_limprichtii
Androsace_stenophylla
Androsace_chamaejasme
Androsace_bungeana
Androsace_minor
Androsace_sempervivoides
Androsace_delavayi
Androsace_spinulifera
Androsace_globifera
Androsace_nortonii
Androsace_barbulata
Androsace_hookeriana
Androsace_erecta
Androsace_axillaris
Androsace_cuscutiformis
Androsace_elatior
Androsace_maxima
Androsace_maxima_torrepandoi
Androsace_filiformis
0.75
0.92
0.16
0.74
0.25
0.57
0.93
0.88
0.22
0.82
0.65
0.93
0.93
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
/Soldanella clade
/Primula clade (collapsed)
/Androsace clade

Fig. S1C

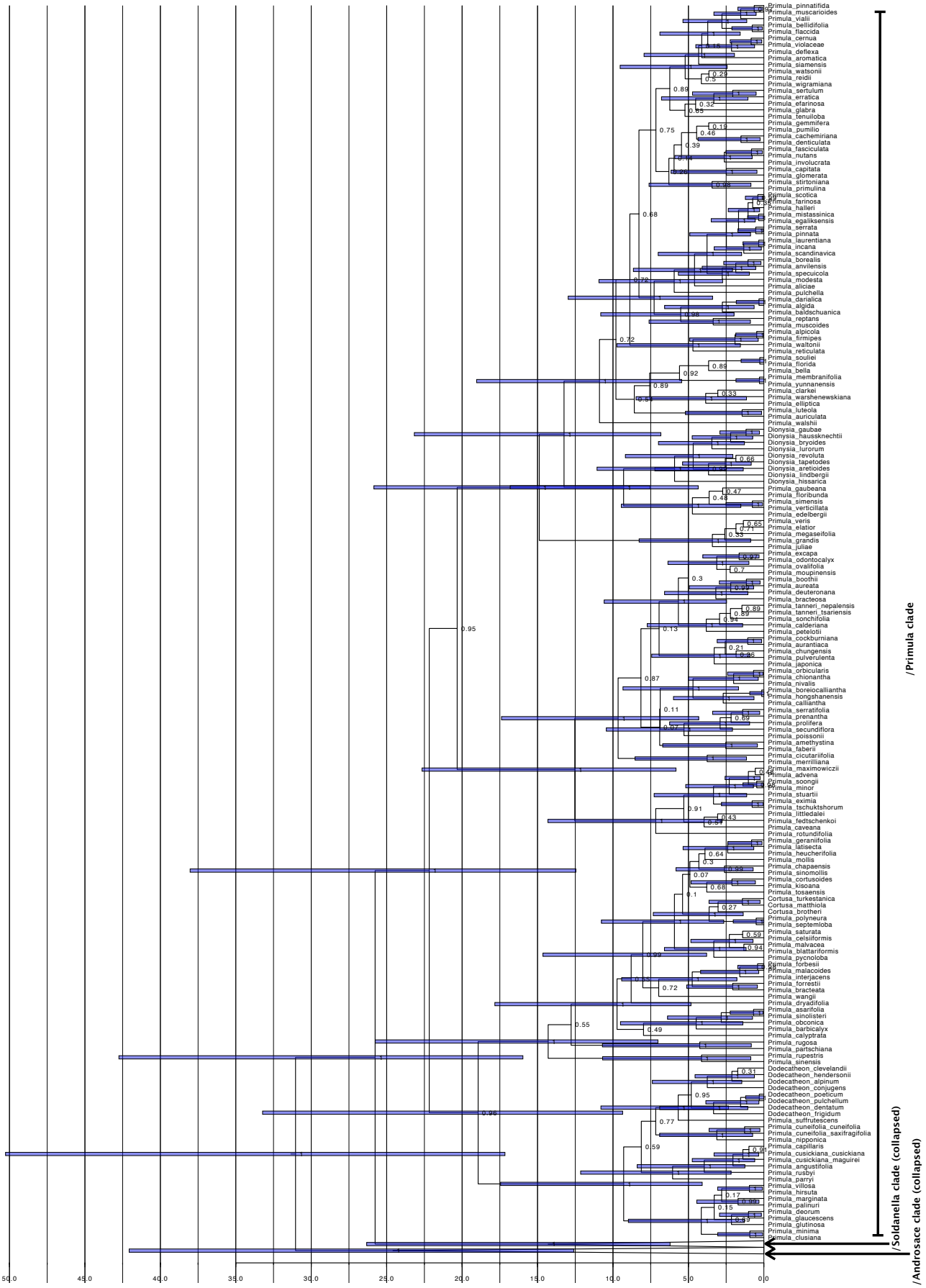
/Primula clade
/Soldanella clade (collapsed)
/Androsace clade (collapsed)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0

Fig. S1C (cont.)

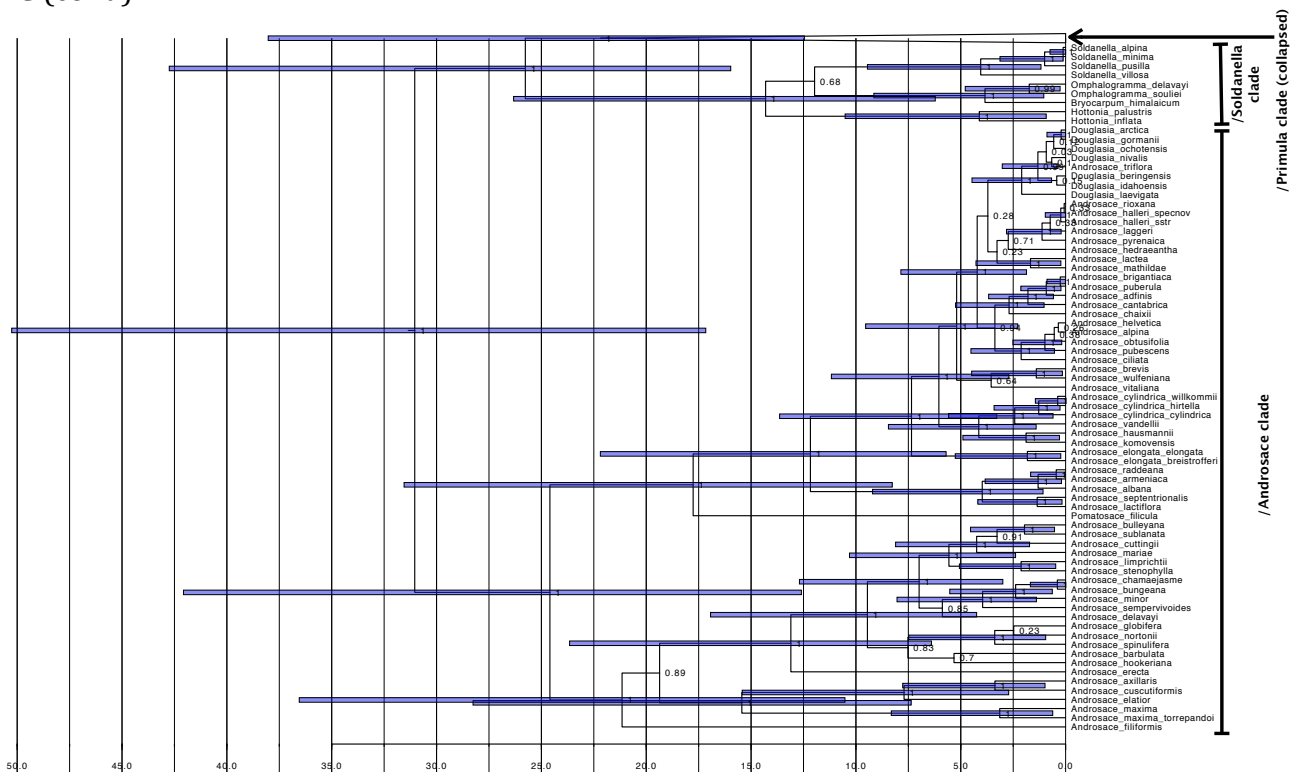

Fig. S1. Phylogenetic relationships within Primulaceae inferred with MrBayes (A) and BEAST using the uncorrelated relaxed lognormal (B) and exponential (C) molecular clock. (A) Majority rule consensus tree of the MrBayes analysis of the Primulacaeae dataset, under the optimal, fully partitioned model. Posterior probability is indicated above branches, clade names are indicated to the right of the tree, scale bar indicates expected number of substitutions per site. First page contains the /Primula clade, including the branch that connects it to the non-heterostylous clades /Soldanella and /Androsace; the /Primula clade is collapsed on the second page. Note that the clades /Primula, /Soldanella and /Androsace received 100% posterior probability, justifying to constrain them as monophyletic in the BEAST analyses. (B) Maximum clade credibility chronogram based on the BEAST dating analysis using the uncorrelated relaxed lognormal molecular clock. Posterior probability is indicated at nodes, clade names are indicated to the right of the tree, scale bar indicates divergence times in millions of years. The interval of 95% highest posterior density of divergence times is given by bars at nodes with >0.95 posterior probability. First page contains the /Primula clade, with the clades /Soldanella and /Androsace collapsed. The /Primula clade is collapsed on the second page. (C) Maximum clade credibility chronogram based on the BEAST dating analysis using the uncorrelated relaxed lognormal molecular clock, annotated as described for Fig. S1B.

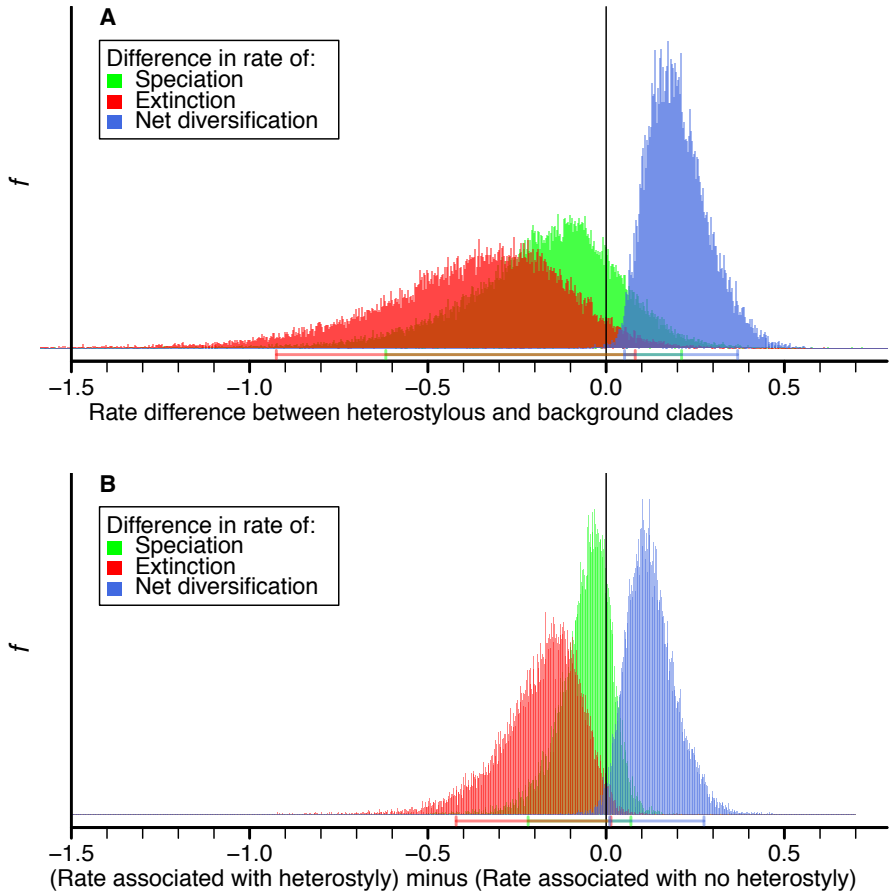

Fig. S2. Frequency distributions of differences in rates of extinction (red), speciation (green), and net diversification (blue) between (A) heterostylous and non-heterostylous clades using BayesRate (Silvestro et al., 2011) and (B) heterostylous and non-heterostylous lineages using BiSSE (Maddison et al. 2007), using the trees from the dating analysis based on the uncorrelated relaxed exponential model. Lines of corresponding colors below the distributions denote the 95% intervals of highest posterior density; intervals that include zero indicate no significant difference in rate. Both types of analyses indicate no significant difference in speciation rates between heterostylous and non-heterostylous lineages, but significantly lower extinction rates (or marginally so in the BiSSE analysis shown in this Figure) , hence, higher diversification rates of heterostylous lineages. BayesFactors for diversification models based on BayesRate analysis: global speciation and global extinction: -17.58; global speciation and clade-specific extinction: 0.00 [i.e. best model]; clade-specific speciation and global extinction: -128.61; clade-specific speciation and clade-specific extinction: -133.77). See Fig. 3 and Table 3 for results of BiSSE and BayesRate analyses based on the dating analysis employing the uncorrelated relaxed exponential model.

Fig. S3

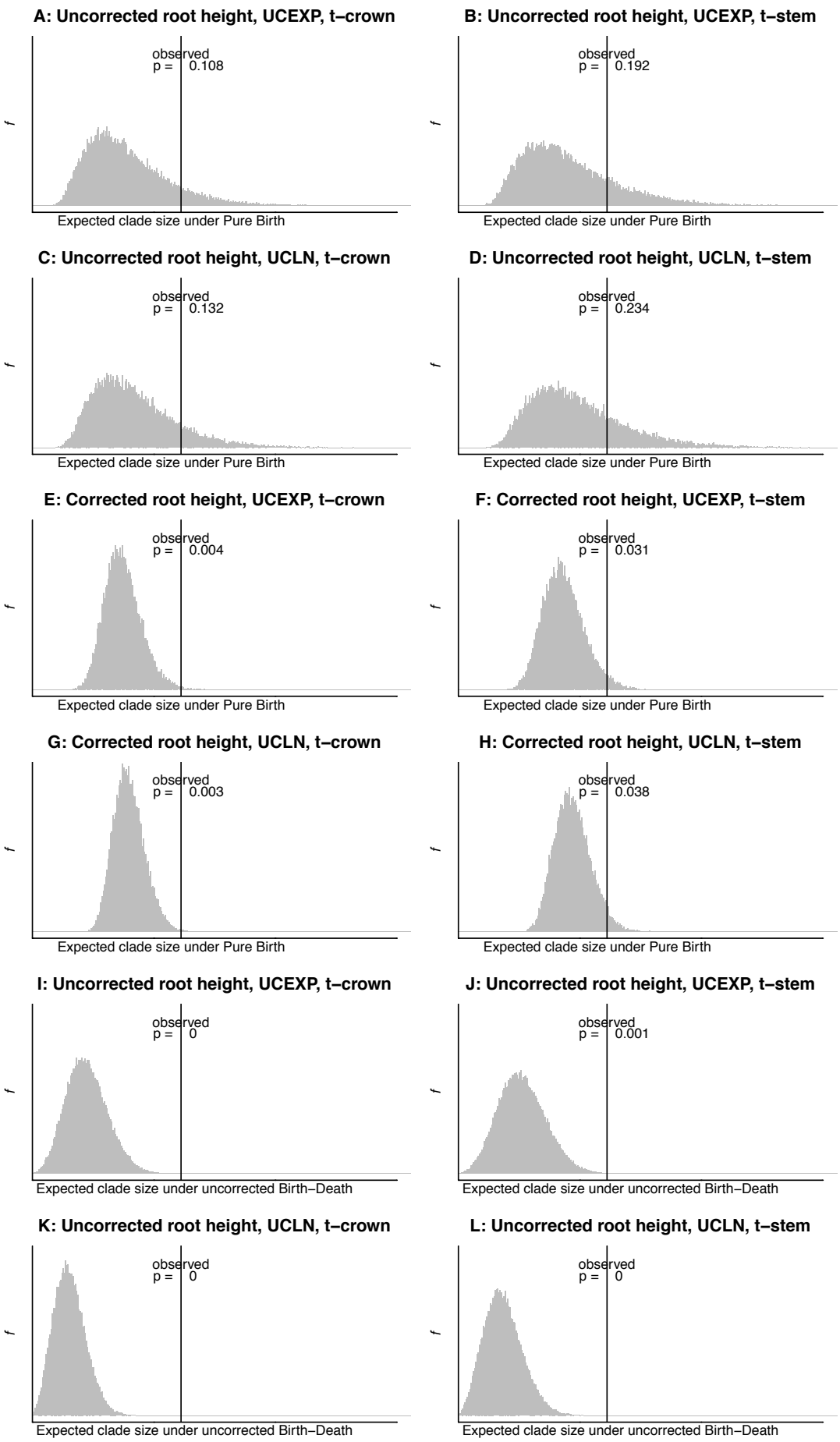
A: Uncorrected root height, UCEXP, t–crown
observed
p = 0.108
f
Expected clade size under Pure Birth
B: Uncorrected root height, UCEXP, t–stem
observed
p = 0.192
f
Expected clade size under Pure Birth
C: Uncorrected root height, UCLN, t–crown
observed
p = 0.132
f
Expected clade size under Pure Birth
D: Uncorrected root height, UCLN, t–stem
observed
p = 0.234
f
Expected clade size under Pure Birth
E: Corrected root height, UCEXP, t–crown
observed
p = 0.004
f
Expected clade size under Pure Birth
F: Corrected root height, UCEXP, t–stem
observed
p = 0.031
f
Expected clade size under Pure Birth
G: Corrected root height, UCLN, t–crown
observed
p = 0.003
f
Expected clade size under Pure Birth
H: Corrected root height, UCLN, t–stem
observed
p = 0.038
f
Expected clade size under Pure Birth
I: Uncorrected root height, UCEXP, t–crown
observed
p = 0
f
Expected clade size under uncorrected Birth–Death
J: Uncorrected root height, UCEXP, t–stem
observed
p = 0.001
f
Expected clade size under uncorrected Birth–Death
K: Uncorrected root height, UCLN, t–crown
observed
p = 0
f
Expected clade size under uncorrected Birth–Death
L: Uncorrected root height, UCLN, t–stem
observed
p = 0
f
Expected clade size under uncorrected Birth–Death

Fig. S3 (cont.)

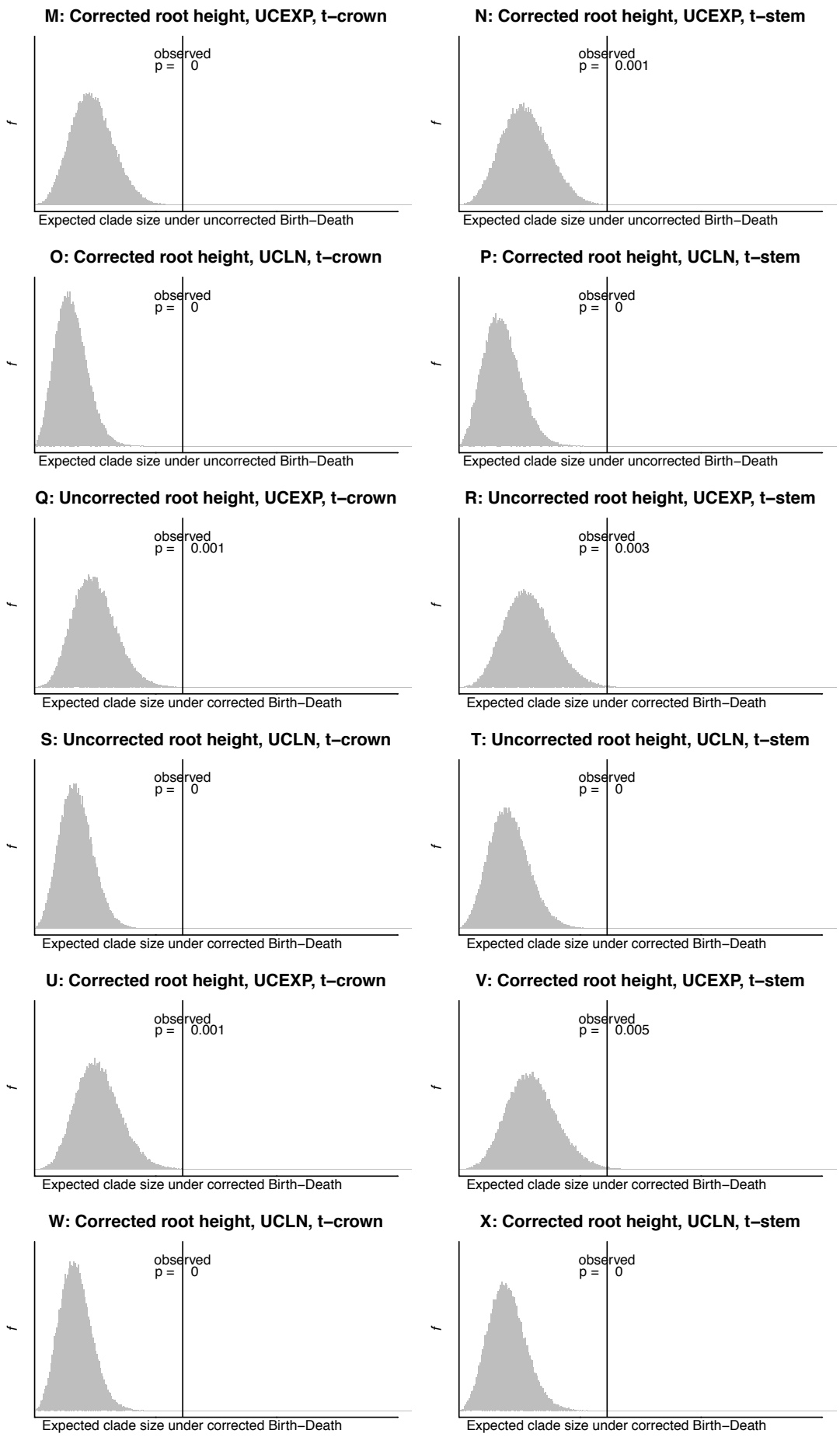
M: Corrected root height, UCEXP, t–crown
observed
p = 0
f
Expected clade size under uncorrected Birth–Death
N: Corrected root height, UCEXP, t–stem
observed
p = 0.001
f
Expected clade size under uncorrected Birth–Death
O: Corrected root height, UCLN, t–crown
observed
p = 0
f
Expected clade size under uncorrected Birth–Death
P: Corrected root height, UCLN, t–stem
observed
p = 0
f
Expected clade size under uncorrected Birth–Death
Q: Uncorrected root height, UCEXP, t–crown
observed
p = 0.001
f
Expected clade size under corrected Birth–Death
R: Uncorrected root height, UCEXP, t–stem
observed
p = 0.003
f
Expected clade size under corrected Birth–Death
S: Uncorrected root height, UCLN, t–crown
observed
p = 0
f
Expected clade size under corrected Birth–Death
T: Uncorrected root height, UCLN, t–stem
observed
p = 0
f
Expected clade size under corrected Birth–Death
U: Corrected root height, UCEXP, t–crown
observed
p = 0.001
f
Expected clade size under corrected Birth–Death
V: Corrected root height, UCEXP, t–stem
observed
p = 0.005
f
Expected clade size under corrected Birth–Death
W: Corrected root height, UCLN, t–crown
observed
p = 0
f
Expected clade size under corrected Birth–Death
X: Corrected root height, UCLN, t–stem
observed
p = 0
f
Expected clade size under corrected Birth–Death

Fig. S3. Frequency distributions of *E(N | r, t)*, the species diversity in the /Primula clade, *N*, expected based on the diversification rate in /Soldanella + /Androsace (i.e. background diversification rate, *r*), and the time since the origin of heterostyly, *t*, using Moore & Donoghue's (2009) "Posterior Predictive Diversity Densities" approach, under 24 analytical scenarios. We calculated *E(N | r, t)* using eight estimates for *t*, and three estimates for *r*, as follows. The background diversification rate, *r*, was estimated under a pure-birth model of diversification in separate BEAST analyses (panels A-H), under a birth-death model of diversification using BayesRate (panels I-P), or under a Birth-Death model of diversification corrected for incomplete taxon sampling using BayesRate (panels Q-X). The time since the origin of heterostyly, *t*, was extracted from dating analyses employing an uncorrelated relaxed exponential model (UCEXP; panels A, B, E, F, I, J, M, N, Q, R, U, V) or employing an uncorrelated relaxed lognormal model (UCLN; panels C, D, G, H, K, L, O, P, S, T, W, X), assuming that heterostyly evolved at the crown node of /Primula (t_{crown}; panels A, C, E, G, I, K, M, O, Q, S, U, W; see node c in Fig. 2) or at the stem node of /Primula (t_{stem}; panels B, D, F, H, J, L, N, P, R, T, V, X; see node b in Fig. 2). The dating analyses to obtain the crown and stem ages of /Primula were performed using the calibration priors as described in the main text (uncorrected root height; panels A, B, C, D, I, J, K, L, Q, R, S, T) or by correcting the root of the Primulaceae phylogeny by fixing it at the point of highest posterior density (i.e. 38.25 Ma; panels E, F, G, H, M, N, O, P, U, V, W, X). To be conservative, we used as observed diversity the number of heterostylous species in /Primula (n=457), rather than the total number of species (see SI Appendix, Table S1, for details on species number per clade). All analytical scenarios indicated that the heterostylous clade is significantly larger than expected based on the background diversification rate, except for the four scenarios that are based on a dating analysis with uncorrected root height combined with a pure-birth model of diversification (panels A-D).

Table S1. Results of ancestral state reconstruction based on the trees obtained under two dating methods for five nodes of interest, indicating for each of four character coding schemes the interval of highest posterior density (HPD) for the node being heterostylous under Bayesian inference using BayesTraits, followed by the probability of heterostyly under Maximum Likelihood in brackets, and the BayesFactor (BF) support for heterostyly as ancestral state, where values >2.3 and <-2.3 indicate significant support for heterostyly or no heterostyly, respectively. Nodes of interest are indicated in the tree of Fig. 2 (main text).

Dating analysis	Node of interest	Scheme 1		Scheme 2		Scheme 3		Scheme 4	
		HPD (ML)	BF	HPD (ML)	BF	HPD (ML)	BF	HPD (ML)	BF
UCEXP	a	0.12-0.79 (0.45)	-0.50	0.18-0.83 (0.51)	0.20	0.25-0.84 (0.54)	0.30	0.28-1.00 (0.83)	1.81
	b	0.62-0.99 (0.88)	0.33	0.66-1.00 (0.89)	0.64	0.68-1.00 (0.88)	1.63	0.72-1.00 (0.97)	2.16
	c	0.98-1.00 (1.00)	8.05	0.98-1.00 (1.00)	8.38	0.96-1.00 (1.00)	8.04	0.99-1.00 (1.00)	10.08
	d	0.01-0.31 (0.11)	-2.66	0.02-0.37 (0.14)	-1.54	0.02-0.38 (0.17)	-1.93	0.00-0.10 (0.03)	-5.30
	e	0.00-0.14 (0.02)	-4.53	0.00-0.14 (0.04)	-4.09	0.00-0.18 (0.06)	-3.90	0.00-0.13 (0.04)	-3.17
UCLN	a	0.10-0.75 (0.41)	-0.49	0.19-0.81 (0.49)	0.09	0.25-0.80 (0.53)	0.28	0.30-1.00 (0.87)	1.61
	b	0.67-0.99 (0.88)	1.27	0.70-1.00 (0.89)	1.55	0.69-1.00 (0.89)	2.21	0.77-1.00 (0.98)	2.97
	c	0.97-1.00 (1.00)	7.60	0.96-1.00 (1.00)	7.44	0.93-1.00 (1.00)	7.25	0.99-1.00 (1.00)	10.63
	d	0.02-0.34 (0.15)	-2.25	0.05-0.40 (0.20)	-1.68	0.08-0.43 (0.24)	-1.67	0.01-0.12 (0.05)	-4.86
	e	0.00-0.12 (0.03)	-4.95	0.01-0.18 (0.06)	-3.88	0.01-0.25 (0.10)	-3.25	0.01-0.14 (0.05)	-3.54

Table S2. Results of analyses to detect shifts in diversification rate using (A) Medusa (Alfaro et al. 2009) and (B) SymmeTree (Chan & Moore 2004). (A) Results of Medusa analyses, indicating the dating analysis on which the input tree was based, diversification model fitted (base model: no rate shifts; optimal model: including rate shifts), number of the nodes where the diversification rate was inferred to change, the Maximum Likelihood estimates of *r* (net diversification) and *epsilon* (relative extinction) for the tree partition, the fit of the model to the data based on AICc value, and the magnitude of the change in diversification rate along the /Primula stem lineage (between nodes b and c; see Fig. 2 of the main text). Note that the difference in AICc strongly supports a model with rate shifts along the tree, rather than the base model without rate shifts. (B) Results of SymmeTree analyses, indicating the nodes where the difference in net diversification rate between the two descending daughter lineages was significant or marginally significant, the value of the shift statistics Δ1 and Δ2 and associated P-values. Results of SymmeTree analyses on trees obtained under both dating analyses were identical. Nodes are numbered according to standard NEXUS tree representation.

A

Dating analysis	Diversification model	Node number	*r*	*epsilon*	AICc	Increase along /Primula stem lineage?
UCEXP	Base model	NA	0.218	0.062	1312.615	NA
	Optimal model	266	0.108	0.403	1292.124	2.18-fold[3]
		503	0.592	0.000		
		268[1, 2]	0.235	0.000		
		327	0.710	0.000		
UCLN	Base model	NA	0.188	0.406	1312.615	NA
	Optimal model	266	0.080	0.516	1199.898	3.46-fold[3]
		474	0.727	0.000		
		268[1, 2]	0.277	0.000		
		280	1.029	0.121		

B

Node number	Shift statistic Δ1	$P_{\Delta 1}$	Shift statistic Δ2	$P_{\Delta 2}$
267*	1.973	0.063	1.786	0.08
285	2.752	0.047	2.485	0.06
335	3.039	0.024	2.773	0.027
338	1.682	0.086	1.576	0.099
345	2.315	0.077	2.079	0.099
438	2.534	0.038	2.303	0.045
471	2.56	0.056	2.303	0.065
473	2.537	0.058	2.303	0.075

Notes:

1: Node 268 represents the most recent common ancestor of all heterostylous species in the /Primula clade (i.e. of >99% of all heterostylous species).

2: A rate shift at node 268, the /Primula crown node, was also recovered in all trees in a sample of 100 trees from the posterior distribution.

3: Calculated using r from position 266 (root) and 268 (/Primula crown node)

Table. S3. Rates of speciation (λ), extinction (μ), and net diversification ($r = \lambda - \mu$) between heterostylous (subscript 1) and non-heterostylous lineages (subscript 0) inferred using BiSSE (Maddison et al. 2007), implemented in Diversitree (FitzJohn 2011). Rates are given as mean ± 1 SE of Maximum Likelihood estimates based on each of 100 trees from the posterior distribution under the UCLN and UCEXP dating analyses. Note the discrepancy between the effect of heterostyly on long time scales (at the family level, i.e. when analyzing all clades jointly), namely increased diversification ($r_1 > r_0$) due to decreased extinction ($\mu_1 < \mu_0$), and the opposite effect of heterostyly on short time scales (at the infra-generic level, i.e. when excluding all data except /Primula), namely decreased diversification ($r_1 < r_0$) due to decreased speciation ($\lambda_1 < \lambda_0$).

Data	λ_0	λ_1	$\boldsymbol{\lambda_1-\lambda_0}$	μ_0	μ_1	$\boldsymbol{\mu_1-\mu_0}$	r_0	r_1	$\boldsymbol{r_1-r_0}$	q_{01}	q_{10}
All clades (UCLN)	0.431 ± 0.011	0.319 ± 0.008	**-0.113** ± 0.004	0.359 ± 0.01	0 ± 0	**-0.359** ± 0.01	0.072 ± 0.002	0.319 ± 0.008	**0.247** ± 0.007	0.005 ± 0	0.06 ± 0.002
/Primula (UCLN)	0.672 ± 0.02	0.191 ± 0.005	**-0.481** ± 0.018	0 ± 0	0.004 ± 0.002	**0.004** ± 0.002	0.672 ± 0.02	0.187 ± 0.005	**-0.486** ± 0.018	0.869 ± 0.043	0.128 ± 0.008
All clades (UCEXP)	0.312 ± 0.01	0.25 ± 0.007	**-0.062** ± 0.004	0.206 ± 0.009	0 ± 0	**-0.206** ± 0.009	0.106 ± 0.003	0.25 ± 0.007	**0.144** ± 0.005	0.007 ± 0	0.044 ± 0.001
/Primula (UCEXP)	0.353 ± 0.013	0.217 ± 0.006	**-0.136** ± 0.009	0 ± 0	0.001 ± 0.001	**0.001** ± 0.001	0.352 ± 0.013	0.216 ± 0.006	**-0.137** ± 0.01	0.321 ± 0.014	0.044 ± 0.002

Table S4. Number of known and sampled species in Primulaceae, indicating the phylogenetic (clade) and taxonomic (genus and section) affinity, the number of species included in recent revisions, the estimated number of species including recently described species, the number of sampled species, the total number of species that are heterostylous, the number of sampled heterostylous species, the numbers of sampled species divided by the total number of species, and the number of sampled heterostylous species divided by the total number of heterostylous species. Note that the proportion of known species that are sampled in this study is similar among the three clades and that the proportion of sampled species that are heterostylous is similar to the proportion of known species that are heterostylous.

Clade	Genus	Section	Number of species in revisions and Flora of China[1]	Estimated total number of species[2]	Number of sampled species	Total number of heterostylous species[3]	Number of sampled heterostylous species[4]	Number of sampled species / Total number of species	Number of sampled heterostylous species / Total number of heterostylous species
/Androsace	*Androsace*	*Aizoidium*	3	3	1	0	0	0.33	NA
/Androsace	*Androsace*	*Andrapis*	17	17	11	0	0	0.65	NA
/Androsace	*Androsace*	*Aretia*	21	22	27	0	0	1.23	NA
/Androsace	*Androsace*	*Chamaejasme*	77	77	15	0	0	0.19	NA
/Androsace	*Androsace*	*Pseudoprimula*	24	24	3	0	0	0.13	NA
/Androsace	*Androsace*	*"Vitaliana"*	1	1	1	1	1	1.00	1.00
/Androsace	*Douglasia*		9	9	7	0	0	0.78	NA
/Androsace	*Pomatosace*		1	1	1	0	0	1.00	NA
/Primula	*Cortusa*		13	8	3	0	0	0.38	NA
/Primula	*Dionysia*		49	49	9	47	9	0.18	0.19
/Primula	*Dodecatheon*		17	17	8	0	0	0.47	NA
/Primula	*Primula*	*Aleuritia*	27	38	16	28	11	0.42	0.39
/Primula	*Primula*	*Amethystina*	8	8	2	8	2	0.25	0.25
/Primula	*Primula*	*Armerina*	14	14	6	10	5	0.43	0.50
/Primula	*Primula*	*Auganthus*	2	2	2	2	2	1.00	1.00
/Primula	*Primula*	*Auricula*	22	22	9	22	9	0.41	0.41
/Primula	*Primula*	*Bullatae*	7	7	2	7	2	0.29	0.29
/Primula	*Primula*	*Capitatae*	2	2	2	2	2	1.00	1.00
/Primula	*Primula*	*Carolinella*	9	11	5	7	4	0.45	0.57
/Primula	*Primula*	*Chartacea*	5	8	1	7	1	0.13	0.14
/Primula	*Primula*	*Cordifoliae*	7	8	3	6	3	0.38	0.50
/Primula	*Primula*	*Cortusoides*	22	27	9	24	7	0.33	0.29
/Primula	*Primula*	*Crystallophlomis*	45	53	13	49	12	0.25	0.24
/Primula	*Primula*	*Cuneifolia*	2	2	3	1	2	1.50	2.00
/Primula	*Primula*	*Davidii*	17	20	3	18	3	0.15	0.17
/Primula	*Primula*	*Denticulata*	12	14	5	14	5	0.36	0.36
/Primula	*Primula*	*Dryadifolia*	4	4	1	4	1	0.25	0.25
/Primula	*Primula*	*Fedtschenkoana*	1	1	1	1	1	1.00	1.00
/Primula	*Primula*	*Glabra*	3	3	1	2	1	0.33	0.50
/Primula	*Primula*	*Malvacea*	5	6	4	6	4	0.67	0.67
/Primula	*Primula*	*Minutissimae*	23	24	7	16	5	0.29	0.31
/Primula	*Primula*	*Monocarpicae*	13	14	4	14	4	0.29	0.29
/Primula	*Primula*	*Muscarioides*	19	20	10	15	7	0.50	0.47
/Primula	*Primula*	*Obconicolisteri*	16	16	4	9	2	0.25	0.22

/Primula	*Primula*	*Oreophlomis*	8	8	5	8	5	0.63	0.63
/Primula	*Primula*	*Parryi*	5	5	6	5	6	1.20	1.20
/Primula	*Primula*	*Petiolares*	28	39	9	37	9	0.23	0.24
/Primula	*Primula*	*Pinnatae*	4	4	2	2	1	0.50	0.50
/Primula	*Primula*	*Primula*	6	6	4	6	4	0.67	0.67
/Primula	*Primula*	*Proliferae*	19	24	10	16	5	0.42	0.31
/Primula	*Primula*	*Pulchella*	14	14	1	14	1	0.07	0.07
/Primula	*Primula*	*Pycnoloba*	1	1	1	1	1	1.00	1.00
/Primula	*Primula*	*Reinii*	4	4	1	4	1	0.25	0.25
/Primula	*Primula*	*Sikkimensis*	9	9	4	8	4	0.44	0.50
/Primula	*Primula*	*Soldanelloides*	18	18	2	17	2	0.11	0.12
/Primula	*Primula*	*Sphondylia*	8	8	5	5	2	0.63	0.40
/Primula	*Primula*	*Sredinskya*	1	1	1	0	0	1.00	NA
/Primula	*Primula*	*Suffrutescens*	1	1	1	1	1	1.00	1.00
/Primula	*Primula*	*Yunnanensis*	15	16	5	14	5	0.31	0.36
/Soldanella	*Soldanella*		16	16	4	0	0	0.25	NA
/Soldanella	*Bryocarpum*		1	1	1	0	0	1.00	NA
/Soldanella	*Hottonia*		2	2	2	1	1	1.00	1.00
/Soldanella	*Omphalogramma*		9	9	2	0	0	0.22	NA
Total of clade /Androsace			**153**	**155**	**66**	**1**	**1**	**0.43**	**1.00**
Total of clade /Soldanella			**28**	**28**	**9**	**1**	**1**	**0.32**	**1.00**
Total of clade /Primula			**505**	**556**	**190**	**457**	**151**	**0.34**	**0.33**
GRAND TOTAL			**690**	**738**	**265**	**459**	**153**	**0.36**	**0.33**

Notes:

1: According to the following major taxonomic revisions: Smith & Lowe, 1997 (*Androsace, Douglasia*); Liden, 2007 (*Dionysia*); Mast & Reveal, 2007 (*Dodecatheon*); Richards, 2003 (*Primula*); Zhang & Kadereit, 2002 (*Soldanella*), Flora of China (Hu & Kelso, 1995; *Bryocarpum*, *Cortusa*, *Omphalogramma*, *Pomatosace, Primula*).

2: For the estimated number of species in *Primula*, we primarily followed Richards (2003), except for Chineese species, where we followed Hu & Kelso (1995), because Richards (2003) does not accept some species shown by Yan et al. (2010) to represent distinct lineages (e.g. *Primula wangii*). The true number of *Primula* species is unknown and may lay somewhere in between Hu & Kelso's and Richards' estimates. Additionally, estimate includes the following recently described species: *Androsace komovensis* Schönsw. & Schneew. in: Taxon 58(2): 547 (544-549; figs.) (2009) (Section *Aretia*); *Androsace kuczerovii* Knjaz. in: Bot. Zhurn. (Moscow & Leningrad) 83(3): 137 (1998) (unclear sectional affiliation, only counted in total of clade /Androsace); *Primula bukukunica* Kovt. in: Bot. Zhurn. (Moscow & Leningrad) 94(12): 1836 (1835-1841; fig. 1-2) (2009) (Section *Aleuritia*); *Primula calyptrata* X.Gong & R.C.Fang in: Novon 13(2): 193 (2003) (Section *Carolinella*); *Primula arunachalensis* S.K.Basak & Maiti in: Acta Phytotax. Geobot. 51(1): 11 (2000) (Section *Chartacea*) *Primula fenghwaiana* C.M.Hu & G.Hao in: Edinburgh J. Bot. 68(2): 298 (-299; fig. 1) (2011) (Section *Chartacea*); *Primula nghialoensis* D.W.H.Rankin in: Curtis's Bot. Mag. 27(2): 138 (132-139; figs. 1-2, pl. 674) (2010) (Section *Chartacea*); *Primula rebeccae* A.J.Richards in: Plantsman n.s., 3(1): 54 (-56; fig.) (2004) (Section *Cordifoliae*); *Primula pskemensis* Lazkov in: Novosti Sist. Vyssh. Rast. 36: 36 (35-38; fig. 4) (2004) (Section *Cortusoides*); *Primula lilacina* A.J.Richards in: Plantsman n.s., 7(2): 123 (-124; fig.) (2008) (Section Muscarioides); Primula bergenioides C.M.Hu & Y.Y.Geng in: Novon 13(2): 196 (2003) (Section *Petiolares*); *Primula lihengiana* C.M.Hu & R.Li in: Ann. Bot. Fenn. 46(2): 130 (-132; fig. 1) (2009) (Section *Petiolares*); *Primula tenuituba* C.M.Hu & Y.Y.Geng in: Novon 13(2): 199 (2003) (Section *Petiolares*).

3: Breeding system information follows taxonomic literature; conflict between Richards (2003) and Hu & Kelso (1995) was judged based on Ernst (1962), if possible. The fraction of all species in *Primula* that are heterostylous was calculated among species of which the breeding system is known. For this table, species that were heterostylous in some but not all populations were counted as 50% heterostylous and 50% non-heterostylous.

4: Scored according to coding scheme 3, that is, species for which heterostyly occurs in some, but not in all populations are not considered heterostylous.

Table S5. Genbank accession numbers for DNA sequence data of the 21 taxa in the Ericales chloroplast DNA dataset that was used to provide a secondary calibration for the Primulaceae dataset. This dataset is a subset of the dataset used by Bremer et al. (2004) to date the orders and families of Asterids, but additionally includes *Androsace*, to be able to date the crown age of Primulaceae, i.e. the split of *Primula* from *Androsace*.

Taxon	*rbc*L gene	*ndh*F gene	*mat*K gene	*trn*V intron	*rps*16 intron	*trn*L intron
Fouquieriaceae *Fouquieria*	L11675	AJ236249	AJ429285	AJ429643	AJ430998	AJ430876
Polemoniaceae *Polemonium*	L11687	AF421070	AJ429292	AJ429649	AJ431004	AJ430882
Lecythidaceae *Barringtionia*	Z80174	AF421044	AJ429286	AJ429644	AJ430999	AJ430877
Ebenaceae *Diospyros*	Z80185	AF130213	AJ430197	AJ429642	AJ430996	AJ430874
Sapotaceae *Manilkara*	L01932	AF213732	AJ429295	AJ429652	AJ431007	AJ430885
Theophrastaceae *Theophrasta*	U96649	AF213762	AJ429307	AJ429663	AJ431018	AJ430895
Myrsinaceae *Myrsina*	U96652	AF213751	AJ429290	AJ429647	AJ431002	AJ430880
Primulaceae *Androsace*	AF395004	AF421114	DQ378429	N/A	FJ786608	AY274947
Primulaceae *Primula*	U96657	AF213757	AJ429293	AJ429650	AJ431005	AJ430883
Pentaphylacaceae *Pentaphylax*	AJ428891	AJ429106	AJ429291	AJ429648	AJ431003	AJ430881
Sladeniaceae *Sladenia*	AJ403004	AF421081	AJ429297	AJ429654	AJ431009	AJ430081
Ternstroemiaceae *Ternstroemia*	Z80211	AF421076	AJ429302	AJ429659	AJ431013	AJ430890
Actinidiaceae *Actinidia*	L01882	AJ236238	AJ429279	AJ429640	AJ430992	AJ430869
Roridulaceae *Roridula*	L01950	AJ236270	AJ429294	AJ429651	AJ431006	AJ430884
Clethraceae *Clethra*	L12609	AJ236242	AJ429281	AJ429526	AJ430994	AJ430871
Cyrillaceae *Cyrilla*	L01900	AF421051	AJ429282	AJ429527	AJ430995	AJ430872
Theaceae *Camellia*	L12602	AF130216	AJ429305	AJ429661	AJ431016	AJ430893
Theaceae *Schima*	Z80208	AF421073	AJ429306	AJ429662	AJ431017	AJ430894
Symplocaceae *Symplocos*	Z80192	AF421074	AJ429301	AJ429658	AJ431012	AJ430889
Styracaceae *Halesia*	Z80190	AF130214	AJ429298	AJ429655	AJ431010	AJ430082
Styracaceae *Styrax*	L12623	AF130215	AJ429300	AJ429657	AJ431011	AJ430888

Table S6. Taxa included in the Primulaceae dataset, indicating genbank accession numbers for the four cpDNA regions included (i.e., *mat*K, *rpl*16, *trn*L, *trn*L-*trn*F) presence of heterostyly ("present", when all reports indicate heterostyly, "absent" when no reports of heterostyly exist, or "both", when reports exist indicating both presence and absence of heterostyly), scoring of the presence of heterostyly under four schemes (present: 1; absent: 0; see main text for rationale of using multiple scoring schemes), and notes describing the scoring for species of which the assignment differed among the four character coding schemes or clarify scoring when literature was ambiguous.

Taxon	Genbank accession numbers					Character coding schemes				
	*mat*K	*rpl*16	*trn*L	*trn*L-*trn*F	Heterostyly present?	Scheme 1	Scheme 2	Scheme 3	Scheme 4	Notes
Androsace adfinis	pending	pending	AY275008	AY275008	absent	0	0	0	0	
Androsace albana	pending	pending	EU655583	EU655583	absent	0	0	0	0	
Androsace alpina	pending	pending	AY274975	AY274975	absent	0	0	0	0	
Androsace armeniaca	pending	pending	pending	pending	absent	0	0	0	0	
Androsace axillaris	pending	pending	AY274949	AY274949	absent	0	0	0	0	
Androsace barbulata	pending	pending	pending	pending	absent	0	0	0	0	
Androsace brevis	pending	pending	AY274963	AY274963	absent	0	0	0	0	
Androsace brigantiaca	pending	pending	EU655591	EU655591	absent	0	0	0	0	
Androsace bulleyana	pending	pending	pending	pending	absent	0	0	0	0	
Androsace bungeana	pending	pending	pending	pending	absent	0	0	0	0	
Androsace cantabrica	pending	pending	AY275009	AY275009	absent	0	0	0	0	
Androsace chaixii	pending	pending	AY275003	AY275003	absent	0	0	0	0	
Androsace chamaejasme	DQ378429	AF402556	AF402437	DQ378838	absent	0	0	0	0	
Androsace ciliata	pending	pending	AY274982	AY274982	absent	0	0	0	0	
Androsace cuscutiformis	pending	pending	pending	pending	absent	0	0	0	0	
Androsace cuttingii	pending	pending	pending	pending	absent	0	0	0	0	
Androsace cylindrica cylindrica	pending	pending	EU655595	EU655595	absent	0	0	0	0	
Androsace cylindrica hirtella	pending	pending	EU655594	EU655594	absent	0	0	0	0	
Androsace cylindrica	pending	pending	AY274987	AY274987	absent	0	0	0	0	
Androsace delavayi	pending	pending	pending	pending	absent	0	0	0	0	
Androsace elatior	pending	pending	pending	pending	absent	0	0	0	0	
Androsace elongata	pending	pending	EU655585	EU655585	absent	0	0	0	0	
Androsace elongata elongata	pending	pending	AY275014	AY275014	absent	0	0	0	0	
Androsace erecta	pending	pending	pending	pending	absent	0	0	0	0	
Androsace filiformis	pending	pending	AY274955	AY274955	absent	0	0	0	0	
Androsace globifera	pending	pending	pending	pending	absent	0	0	0	0	
Androsace halleri specnov	pending	pending	AY275013	AY275013	absent	0	0	0	0	
Androsace halleri sstr	pending	pending	EU655587	EU655587	absent	0	0	0	0	
Androsace hausmannii	pending	pending	AY274984	AY274984	absent	0	0	0	0	

Androsace hedraeantha	pending	pending	pending	pending	absent	0	0	0	0	
Androsace helvetica	pending	pending	AY274981	AY274981	absent	0	0	0	0	
Androsace hookeriana	pending	pending	pending	pending	absent	0	0	0	0	
Androsace komovensis	pending	pending	EU655596	EU655596	absent	0	0	0	0	
Androsace lactea	pending	pending	AY274986	AY274986	absent	0	0	0	0	
Androsace lactiflora	pending	pending	EU655582	EU655582	absent	0	0	0	0	
Androsace laggeri	pending	pending	AY275012	AY275012	absent	0	0	0	0	
Androsace limprichtii	pending	pending	pending	pending	absent	0	0	0	0	
Androsace mariae	pending	pending	pending	pending	absent	0	0	0	0	
Androsace mathildae	pending	pending	EU655598	EU655598	absent	0	0	0	0	
Androsace maxima	pending	pending	AY274957	AY274957	absent	0	0	0	0	
Androsace maxima	pending	pending	pending	pending	absent	0	0	0	0	
Androsace minor	pending	pending	pending	pending	absent	0	0	0	0	
Androsace nortonii	pending	pending	pending	pending	absent	0	0	0	0	
Androsace obtusifolia	pending	pending	AY274971	AY274971	absent	0	0	0	0	
Androsace puberula	pending	pending	AY275006	AY275006	absent	0	0	0	0	
Androsace pubescens	pending	pending	AY274979	AY274979	absent	0	0	0	0	
Androsace pyrenaica	pending	pending	AY274977	AY274977	absent	0	0	0	0	
Androsace raddeana	pending	pending	AY274960	AY274960	absent	0	0	0	0	
Androsace rioxana	pending	pending	AY275004	AY275004	absent	0	0	0	0	
Androsace sempervivoides	AY647535	AF402555	AF402436	DQ378837	absent	0	0	0	0	
Androsace septentrionalis	pending	pending	AY274959	AY274959	absent	0	0	0	0	
Androsace spinulifera	pending	pending	pending	pending	absent	0	0	0	0	
Androsace stenophylla	pending	pending	pending	pending	absent	0	0	0	0	
Androsace sublanata	FJ828637	N/A	FJ794240	FJ794240	absent	0	0	0	0	
Androsace triflora	pending	pending	pending	pending	absent	0	0	0	0	
Androsace vandellii	pending	pending	AY274968	AY274968	absent	0	0	0	0	
Androsace vitaliana	pending	pending	AY274966	AY274966	present	1	1	1	0	1
Androsace wulfeniana	pending	pending	AY274962	AY274962	absent	0	0	0	0	
Bryocarpum himalaicum	DQ378424	DQ378516	DQ378605	DQ378830	absent	0	0	0	0	
Cortusa brotheri	DQ378422	DQ378513	DQ378602	DQ378827	absent	0	0	0	0	
Cortusa matthiola	AY647522	AY528555	AY647667	AY647737	absent	0	0	0	0	
Cortusa turkestanica	DQ378421	DQ378512	DQ378601	DQ378826	absent	0	0	0	0	
Dionysia aretioides	DQ378298	DQ378434	DQ378523	DQ378703	present	1	1	1	1	
Dionysia bryoides	pending	pending	pending	pending	present	1	1	1	1	
Dionysia gaubae	pending	pending	pending	pending	present	1	1	1	1	
Dionysia haussknechtii	pending	pending	pending	pending	present	1	1	1	1	
Dionysia hissarica	DQ378299	DQ378435	DQ378524	DQ378704	present	1	1	1	1	
Dionysia lindbergii	pending	pending	pending	pending	present	1	1	1	1	

Dionysia lurorum	pending	pending	pending	pending	present	1	1	1	1	
Dionysia revoluta	pending	pending	pending	pending	present	1	1	1	1	
Dionysia tapetodes	DQ378300	DQ378436	DQ378525	DQ378705	present	1	1	1	1	
Dodecatheon alpinum	AY647475	AY528520	AY647620	AY647690	absent	0	0	0	0	
Dodecatheon clevelandii	AY647465	AY528510	AY647610	AY647680	absent	0	0	0	0	
Dodecatheon conjugens	AY647469	AY528514	AY647614	AY647684	absent	0	0	0	0	
Dodecatheon dentatum	AY647485	AY528530	AY647630	AY647700	absent	0	0	0	0	
Dodecatheon frigidum	AY647471	AY528516	AY647616	AY647686	absent	0	0	0	0	
Dodecatheon hendersonii	AY647462	AY528507	AY647607	AY647677	absent	0	0	0	0	
Dodecatheon poeticum	AY647488	AY528533	AY647633	AY647703	absent	0	0	0	0	
Dodecatheon pulchellum	AY647478	AY528523	AY647623	AY647693	absent	0	0	0	0	
Douglasia arctica	pending	pending	pending	pending	absent	0	0	0	0	
Douglasia beringensis	pending	pending	pending	pending	absent	0	0	0	0	
Douglasia gormanii	pending	pending	pending	pending	absent	0	0	0	0	
Douglasia idahoensis	pending	pending	pending	pending	absent	0	0	0	0	
Douglasia laevigata	pending	pending	pending	pending	absent	0	0	0	0	
Douglasia nivalis	pending	pending	pending	pending	absent	0	0	0	0	
Douglasia ochotensis	pending	pending	pending	pending	absent	0	0	0	0	
Hottonia inflata	DQ378428	DQ378520	DQ378609	DQ378836	absent	0	0	0	0	
Hottonia palustris	AY647534	AF402554	AF402435	DQ378835	present	1	1	1	0	2
Omphalogramma delavayi	DQ378423	DQ378514	DQ378603	DQ378828	absent	0	0	0	0	
Omphalogramma souliei	AY647532	DQ378515	DQ378604	DQ378829	absent	0	0	0	0	
Pomatosace filicula	DQ378431	AF402559	AF402440	DQ378841	absent	0	0	0	0	
Primula advena	DQ378396	AF402525	AF402405	DQ378801	present	1	1	1	1	
Primula algida	DQ378340	AF402468	AF402350	DQ378745	present	1	1	1	1	
Primula aliciae	DQ378303	DQ378438	DQ378527	DQ378708	present	1	1	1	1	
Primula alpicola	FJ828606	N/A	FJ794205	FJ794205	present	1	1	1	1	
Primula amethystina	AY647523	AY528556	AY647668	AY647738	present	1	1	1	1	
Primula angustifolia	AY647514	AF402536	AY647659	AY647729	present	1	1	1	1	
Primula anvilensis	DQ378355	DQ378469	DQ378558	DQ378760	present	1	1	1	1	
Primula aromatica	FJ828630	N/A	FJ794233	FJ794233	present	1	1	1	1	
Primula asarifolia	DQ378405	DQ378502	DQ378591	DQ378810	present	1	1	1	1	
Primula aurantiaca	DQ378378	DQ378481	DQ378570	DQ378783	present	1	1	1	1	
Primula aureata	DQ378375	DQ378478	DQ378567	DQ378780	present	1	1	1	1	
Primula auriculata	DQ378310	AF402462	AF402344	DQ378715	present	1	1	1	1	
Primula baldschuanica	DQ378342	DQ378464	DQ378553	DQ378747	present	1	1	1	1	
Primula barbicalyx	FJ828647	N/A	FJ794251	FJ794251	present	1	1	1	1	
Primula bella	FJ828600	N/A	FJ794198	FJ794198	present	1	1	1	1	
Primula bellidifolia	DQ378312	DQ378442	DQ378531	DQ378717	both	1	0	0	0	3

Primula blattariformis	FJ828654	N/A	FJ794261	FJ794261	present	1	1	1	1	
Primula boothii	DQ378370	DQ378475	DQ378564	DQ378775	present	1	1	1	1	
Primula borealis	AY647527	AF402488	AY647672	AY647742	present	1	1	1	1	
Primula boreiocalliantha	FJ828620	N/A	FJ794220	FJ794220	present	1	1	1	1	
Primula bracteata	DQ378409	AF402548	AF402429	DQ378814	present	1	1	1	1	
Primula bracteosa	DQ378371	DQ378476	DQ378565	DQ378776	present	1	1	1	1	
Primula cachemiriana	DQ378325	DQ378452	DQ378541	DQ378730	present	1	1	1	1	
Primula calderiana	DQ378374	AF402514	AF402394	DQ378779	present	1	1	1	1	
Primula calliantha	DQ378397	DQ378496	DQ378585	DQ378802	present	1	1	1	1	
Primula calyptrata	FJ828646	N/A	FJ794250	FJ794250	absent	0	0	0	0	
Primula capillaris	AY647519	AY528554	AY647664	AY647734	present	1	1	1	1	
Primula capitata	DQ378326	DQ378453	DQ378542	DQ378731	present	1	1	1	1	
Primula caveana	DQ378400	DQ378498	DQ378587	DQ378805	present	1	1	1	1	
Primula celsiiformis	FJ828607	N/A	FJ794206	FJ794206	present	1	1	1	1	
Primula cernua	DQ378317	DQ378446	DQ378535	DQ378722	present	1	1	1	1	
Primula chapaensis	FJ828645	N/A	FJ794249	FJ794249	present	1	1	1	1	
Primula chionantha	DQ378387	DQ378488	DQ378577	DQ378792	present	1	1	1	1	
Primula chungensis	DQ378382	DQ378484	DQ378573	DQ378787	both	1	0	0	0	4
Primula cicutariifolia	DQ378366	DQ378471	DQ378560	DQ378771	absent	0	0	0	0	
Primula clarkei	DQ378307	AF402460	AF402342	DQ378712	present	1	1	1	1	
Primula clusiana	AY647490	AY528534	AY647635	AY647705	present	1	1	1	1	
Primula cockburniana	DQ378380	DQ378483	DQ378572	DQ378785	absent	0	0	0	0	
Primula cortusoides	DQ378412	DQ378505	DQ378594	DQ378817	present	1	1	1	1	
Primula cuneifolia cuneifolia	AY647502	AY528542	AY647647	AY647717	present	1	1	1	1	
Primula cuneifolia	AY647506	AY528545	AY647651	AY647721	absent	0	0	0	0	
Primula cusickiana cusickiana	AY647515	AY528550	AY647660	AY647730	present	1	1	1	1	
Primula cusickiana maguirei	AY647516	AY528551	AY647661	AY647731	present	1	1	1	1	
Primula darialica	DQ378341	AF402470	AF402352	DQ378746	present	1	1	1	1	
Primula deflexa	DQ378315	DQ378444	DQ378533	DQ378720	present	1	1	1	1	
Primula denticulata	DQ378323	DQ378450	DQ378539	DQ378728	present	1	1	1	1	
Primula deorum	AY647497	AF402531	AY647642	AY647712	present	1	1	1	1	
Primula deuteronana	DQ378372	DQ378477	DQ378566	DQ378777	present	1	1	1	1	
Primula dryadifolia	DQ378406	AF402551	AF402432	DQ378811	present	1	1	1	1	
Primula edelbergii	AY647528	AF402452	AY647673	AY647743	present	1	1	1	1	
Primula efarinosa	FJ828616	N/A	FJ794216	FJ794216	present	1	1	1	1	
Primula egaliksensis	DQ378349	AF402481	AF402363	DQ378754	absent	0	0	0	0	
Primula elatior	DQ378361	AF402504	AF402384	DQ378766	present	1	1	1	1	
Primula elliptica	DQ378306	AF402459	AF402341	DQ378711	present	1	1	1	1	
Primula erratica	DQ378322	AF402471	AF402353	DQ378727	present	1	1	1	1	

Primula excapa	DQ378367	DQ378472	DQ378561	DQ378772	present	1	1	1	1	
Primula eximia	AY647525	AF402522	AY647670	AY647740	absent	0	0	0	0	
Primula faberii	AY647524	AY528557	AY647669	AY647739	present	1	1	1	1	
Primula farinosa	DQ378345	AF402474	AF402356	DQ378750	present	1	1	1	1	
Primula fasciculata	DQ378329	DQ378455	DQ378544	DQ378734	present	1	1	1	1	
Primula fedtschenkoi	DQ378399	AF402526	AF402406	DQ378804	present	1	1	1	1	
Primula firmipes	DQ378360	AF402502	AF402382	DQ378765	present	1	1	1	1	
Primula flaccida	DQ378318	DQ378447	DQ378536	DQ378723	present	1	1	1	1	
Primula floribunda	DQ378296	AF402454	AF402336	DQ378701	both	1	1	0	1	5
Primula florida	DQ378302	DQ378437	DQ378526	DQ378707	present	1	1	1	1	
Primula forbesii	AY647520	AF402540	AY647665	AY647735	present	1	1	1	1	
Primula forrestii	DQ378410	AF402549	AF402430	DQ378815	present	1	1	1	1	
Primula gaubeana	DQ378297	DQ378433	DQ378522	DQ378702	present	1	1	1	1	
Primula gemmifera	DQ378332	AF402495	AF402375	DQ378737	present	1	1	1	1	
Primula geraniifolia	DQ378417	AF402546	AF402426	DQ378822	present	1	1	1	1	
Primula glabra	DQ378331	DQ378457	DQ378546	DQ378736	present	1	1	1	1	
Primula glaucescens	pending	pending	pending	pending	present	1	1	1	1	
Primula glomerata	DQ378324	DQ378451	DQ378540	DQ378729	present	1	1	1	1	
Primula glutinosa	AY647495	AF402533	AY647640	AY647710	present	1	1	1	1	
Primula grandis	AY647531	AF402505	AY647676	AY647746	absent	0	0	0	0	
Primula halleri	pending	pending	pending	pending	absent	0	0	0	0	
Primula heucherifolia	DQ378415	DQ378508	DQ378597	DQ378820	present	1	1	1	1	
Primula hirsuta	AY647499	AY528540	AY647644	AY647714	present	1	1	1	1	
Primula hongshanensis	DQ378391	DQ378492	DQ378581	DQ378796	present	1	1	1	1	
Primula incana	DQ378347	AF402478	AF402360	DQ378752	absent	0	0	0	0	
Primula interjacens	FJ828610	N/A	FJ794209	FJ794209	present	1	1	1	1	
Primula involucrata	DQ378328	DQ378454	DQ378543	DQ378733	present	1	1	1	1	
Primula japonica	DQ378379	DQ378482	DQ378571	DQ378784	absent	0	0	0	0	
Primula juliae	DQ378364	AF402508	AF402388	DQ378769	present	1	1	1	1	
Primula kisoana	DQ378414	DQ378507	DQ378596	DQ378819	present	1	1	1	1	
Primula latisecta	DQ378416	DQ378509	DQ378598	DQ378821	present	1	1	1	1	
Primula laurentiana	DQ378348	AF402479	AF402361	DQ378753	absent	0	0	0	0	
Primula littledalei	DQ378401	DQ378499	DQ378588	DQ378806	present	1	1	1	1	
Primula luteola	DQ378309	AF402461	AF402343	DQ378714	present	1	1	1	1	
Primula malacoides	DQ378408	AF402541	AF402421	DQ378813	present	1	1	1	1	
Primula malvacea	FJ828651	N/A	FJ794257	FJ794257	present	1	1	1	1	
Primula marginata	AY647492	AF402530	AY647637	AY647707	present	1	1	1	1	
Primula maximowiczii	DQ378398	DQ378497	DQ378586	DQ378803	present	1	1	1	1	
Primula megaseifolia	DQ378363	AF402507	AF402387	DQ378768	present	1	1	1	1	

Primula membranifolia	DQ378304	AF402458	AF402340	DQ378709	present	1	1	1	1	
Primula merrilliana	FJ828589	N/A	FJ794196	FJ794196	present	1	1	1	1	
Primula minima	AY647494	AY528537	AY647639	AY647709	present	1	1	1	1	
Primula minor	DQ378394	DQ378495	DQ378584	DQ378799	present	1	1	1	1	
Primula mistassinica	DQ378352	AF402485	AF402367	DQ378757	present	1	1	1	1	
Primula modesta	DQ378357	AF402490	AF402371	DQ378762	present	1	1	1	1	
Primula mollis	DQ378418	AF402547	AF402427	DQ378823	both	1	0	0	0	6
Primula moupinensis	FJ828614	N/A	FJ794214	FJ794214	present	1	1	1	1	
Primula muscarioides	DQ378311	DQ378441	DQ378530	DQ378716	present	1	1	1	1	
Primula muscoides	DQ378337	DQ378461	DQ378550	DQ378742	absent	0	0	0	0	
Primula nipponica	AY647508	AY528546	AY647653	AY647723	present	1	1	1	1	
Primula nivalis	DQ378390	DQ378490	DQ378579	DQ378794	present	1	1	1	1	
Primula nutans	AY647526	AF402494	AY647671	AY647741	present	1	1	1	1	
Primula obconica	DQ378403	AF402542	AF402422	DQ378808	both	1	1	0	1	7
Primula odontocalyx	DQ378368	DQ378473	DQ378562	DQ378773	present	1	1	1	1	
Primula orbicularis	DQ378388	DQ378489	DQ378578	DQ378793	present	1	1	1	1	
Primula ovalifolia	FJ828605	N/A	FJ794204	FJ794204	present	1	1	1	1	
Primula palinuri	AY647489	AF402532	AY647634	AY647704	present	1	1	1	1	
Primula parryi	AY647512	AY528548	AY647657	AY647727	present	1	1	1	1	
Primula partschiana	FJ828593	N/A	FJ794190	FJ794190	present	1	1	1	1	
Primula petelotii	DQ378369	DQ378474	DQ378563	DQ378774	present	1	1	1	1	
Primula pinnata	DQ378344	DQ378466	DQ378555	DQ378749	present	1	1	1	1	
Primula pinnatifida	DQ378316	DQ378445	DQ378534	DQ378721	present	1	1	1	1	
Primula poissonii	FJ828619	N/A	FJ794219	FJ794219	present	1	1	1	1	
Primula polyneura	FJ828627	N/A	FJ794227	FJ794227	present	1	1	1	1	
Primula prenantha	DQ378383	AF402519	AF402399	DQ378788	absent	0	0	0	0	
Primula primulina	DQ378335	DQ378460	DQ378549	DQ378740	present	1	1	1	1	
Primula prolifera	DQ378384	DQ378485	DQ378574	DQ378789	both	1	1	0	1	8
Primula pulchella	DQ378339	DQ378463	DQ378552	DQ378744	present	1	1	1	1	
Primula pulverulenta	DQ378385	DQ378486	DQ378575	DQ378790	present	1	1	1	1	
Primula pumilio	DQ378327	AF402493	AF402373	DQ378732	present	1	1	1	1	
Primula pycnoloba	FJ828612	N/A	FJ794212	FJ794212	present	1	1	1	1	
Primula reidii	DQ378320	AF402467	AF402349	DQ378725	present	1	1	1	1	
Primula reptans	DQ378336	AF402496	AF402376	DQ378741	present	1	1	1	1	
Primula reticulata	DQ378358	DQ378470	DQ378559	DQ378763	present	1	1	1	1	
Primula rotundifolia	DQ378402	DQ378500	DQ378589	DQ378807	present	1	1	1	1	
Primula rugosa	FJ828644	N/A	FJ794248	FJ794248	present	1	1	1	1	
Primula rupestris	FJ828585	N/A	FJ794182	FJ794182	present	1	1	1	1	
Primula rusbyi	AY647513	AY528549	AY647658	AY647728	present	1	1	1	1	

Primula saturata	FJ828650	N/A	FJ794256	FJ794256	present	1	1	1	1	
Primula scandinavica	DQ378351	AF402483	AF402365	DQ378756	absent	0	0	0	0	
Primula scotica	pending	pending	pending	pending	absent	0	0	0	0	
Primula secundiflora	FJ828613	N/A	FJ794213	FJ794213	present	1	1	1	1	
Primula septemloba	FJ828656	N/A	FJ794263	FJ794263	absent	0	0	0	0	
Primula serrata	DQ378343	DQ378465	DQ378554	DQ378748	present	1	1	1	1	
Primula serratifolia	FJ828617	N/A	FJ828589	FJ828589	present	1	1	1	1	
Primula sertulum	FJ828604	N/A	FJ794203	FJ794203	present	1	1	1	1	
Primula siamensis	DQ378319	DQ378448	DQ378537	DQ378724	present	1	1	1	1	9
Primula simensis	DQ378295	DQ378432	DQ378521	DQ378700	absent	0	0	0	0	
Primula sinensis	FJ828584	N/A	FJ794181	FJ794181	present	1	1	1	1	
Primula sinolisteri	DQ378404	DQ378501	DQ378590	DQ378809	both	1	1	0	1	10
Primula sinomollis	FJ828634	N/A	FJ794237	FJ794237	present	1	1	1	1	
Primula sonchifolia	DQ378373	AF402513	AF402393	DQ378778	present	1	1	1	1	
Primula soongii	DQ378393	DQ378494	DQ378583	DQ378798	present	1	1	1	1	
Primula souliei	DQ378305	DQ378439	DQ378528	DQ378710	present	1	1	1	1	
Primula specuicola	DQ378354	AF402487	AF402368	DQ378759	present	1	1	1	1	
Primula stirtoniana	DQ378334	DQ378459	DQ378548	DQ378739	present	1	1	1	1	11
Primula stuartii	DQ378392	DQ378493	DQ378582	DQ378797	present	1	1	1	1	
Primula suffrutescens	AY647510	AY528547	AY647655	AY647725	present	1	1	1	1	
Primula tanneri nepalensis	DQ378376	DQ378479	DQ378568	DQ378781	present	1	1	1	1	
Primula tanneri tsariensis	DQ378377	DQ378480	DQ378569	DQ378782	present	1	1	1	1	
Primula tenuiloba	DQ378338	DQ378462	DQ378551	DQ378743	present	1	1	1	1	
Primula tosaensis	DQ378411	DQ378504	DQ378593	DQ378816	present	1	1	1	1	
Primula tschuktshorum	DQ378395	AF402523	AF402403	DQ378800	present	1	1	1	1	
Primula veris	AY647530	AF402503	AY647675	AY647745	present	1	1	1	1	
Primula verticillata	DQ378294	AF402453	AF402335	DQ378699	absent	0	0	0	0	
Primula vialii	DQ378313	AF402466	AF402348	DQ378718	present	1	1	1	1	
Primula villosa	pending	pending	pending	pending	present	1	1	1	1	
Primula violaceae	pending	pending	pending	pending	present	1	1	1	1	
Primula walshii	pending	pending	pending	pending	present	1	1	1	1	
Primula waltonii	DQ378359	AF402500	AF402380	DQ378764	present	1	1	1	1	
Primula wangii	FJ828648	N/A	FJ794252	FJ794252	present	1	1	1	1	
Primula warshenewskiana	DQ378308	DQ378440	DQ378529	DQ378713	present	1	1	1	1	
Primula watsonii	DQ378314	DQ378443	DQ378532	DQ378719	both	1	0	0	0	12
Primula wigramiana	DQ378321	DQ378449	DQ378538	DQ378726	present	1	1	1	1	
Primula yunnanensis	DQ378301	AF402457	AF402339	DQ378706	present	1	1	1	1	
Soldanella alpina	DQ378425	DQ378517	DQ378606	DQ378832	absent	0	0	0	0	
Soldanella minima	DQ378426	DQ378518	DQ378607	DQ378833	absent	0	0	0	0	

Soldanella pusilla	AY647533	AF402553	AF402434	DQ378831	absent	0	0	0	0
Soldanella villosa	DQ378427	DQ378519	DQ378608	DQ378834	absent	0	0	0	0

Notes:

1: Schaeppi (1935; Berichte der Schweizerischen Botanische Gesellschaft 44: 109-132) investigated this species thoroughly and concluded that although the position of the stigma is strongly polymorphic, the position of the anthers is only slightly different between morphs. This finding was confirmed in personal observations (R. Kellenberger & J. M. de Vos). Therefore, we score this species as non-heterostylous in the scoring scheme 4.

2: The short-style morpho of *Hottonia palustris* has anthers positioned well above the corolla, with filaments that are partially free, whereas other heterostylous species have a narrow floral tube to which filaments are fused, concealing sexual organs within (Schaeppi, 1934). Hence, we score this species as non-heterostylous in scoring scheme 4.

3: Among 46 investigated plants Ernst (1962: 78) observed plants with and without heterostyly, but the majority (26) could not be unambiguously assigned to either one, because sexual organ positions appear to be extremely variable in this species, also among plants that seem heterostylous at first glance. Hence, this species displays does not display clear heterostyly in most populations and was therefore coded as non-heterostylous in scheme 2.

4: Ernst (1938: 140-149) showed that populations of this species can be heterostylous, non-heterostylous or consist of a mixture of either floral morph and a non-heterostylous form. Scoring under scheme 2 is thus as non- heterostylous.

5: Ernst (1962: 71) investigated 165 plants in the herbaria Edinburgh, Kew, and Calcutta, and of the 147 plants that had well-preserved flowers, 138 clearly displayed heterostyly. Scoring in scheme 2 is therefore heterostylous.

6: According to Ernst (1959, Archiv Julius Klaus Stiftung 34(1): 74-78) heterostyly is absent in most of the herbarium material of this species; those few collections showing heterostyly also differ in other characters from the non-heterostylous material, which suggests that the heterostylous material may actually represent a different species. Therefore, scoring under scheme 2 is thus as non- heterostylous.

7: Ernst (1962: 82) classifies this species as heterostylous, in which only occasionally aberrant forms occur, as of 120 investigated flowers, only 2 lacked heterostyly. Hence, scoring under scheme 2 is thus as heterostylous.

8: *P. prolifera* has an extremely complex taxonomic history and has a remarkably disjunct distribution, with heterostylous populations in the Eastern Himalaya, and non-heterostylous populations in high mountains on Java and Sumatra (Indonesia) as well as peninsular Malaysia (Richards 2003, Bentvelzen 1962: Fl. Males. 1(6):189-191). There are no accounts of populations in the Himalaya that lack heterostyly, nor has heterostyly been reported from Malaysia or Indonesia. Scoring in scheme 2 is as heterostylous, because the sampled material in this study comes from the Himalaya, and the complex taxonomic history may suggest that disjunct material is distantly related.

9: Richards states the species is identical to *P. spicata* except in leaf characters, *P. spicata* differs only in habit and flower colour from *P. flaccida*, *P. flaccida* is claimed to be heterostylous. Not discussed in Ernst (1962).

10: Ernst (1962) classifies this species as heterostylous but ignores *var. aspera* which lacks heterostyly according to Richards (2003). Because the material used in our study does not represent *var. aspera*, The species was therefore scored as heterostylous in character coding scheme 2.

11: According to Ernst (1962: 75), this species is heterostylous.

12: Ernst (1962: 80) states that 9 of 11 investigated herbarium sheets contained 36 plants lacking heterostyly; 2 herbarium sheets contained mixtures of heterostylous morph and plants that lacked heterostyly. The species was scored as non-heterostylous in character coding scheme 2, because heterostyly appears to be rare in this species.

CHAPTER III: SMALL AND UGLY? A QUANTITATIVE, COMPARATIVE EVALUATION OF THE "SELFING SYNDROME" IN HETEROSTYLOUS AND HOMOSTYLOUS PRIMROSES

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Abstract

One of the most common transitions in plant evolution, the shift from outcrossing to increased selfing after the loss of self-incompatibility, is typically associated with changes in multiple floral characters, termed the selfing syndrome, notably including a reduction of floral size. However, it is unclear what aspects of evolutionary trajectories of floral morphology (e.g. inferred selective optima) change with a shift toward increased selfing and whether there are differences among traits. Here, we use recently developed comparative methods to study quantitative effects of losses of self-incompatibility on four floral traits, as exemplified by nine independent transitions from heterostyly to homostyly among 126 Primrose species, a classic system for the evolution of selfing. We find similar variability among heterostylous and homostylous flowers, but contrasting patterns among traits: homostylous flowers are smaller in some but not all respects. Patterns in pollination-related traits are best explained by a marked increase in the intensity of stochastic fluctuations of evolutionary trajectories associated with losing heterostyly, contradicting the general assumption that floral-morphological changes in selfing species are primarily driven by shifted optima of resource allocation. These results are congruent with an increased importance of drift for evolutionary trajectories of floral morphology after the loss of self-incompatibility.

Introduction

The loss of self-incompatibility (i.e., postpollination prezygotic mechanisms that prevent self-fertilization; Igic et al. 2008) is widely acknowledged as one of the most frequent transitions in plant evolution (Stebbins 1950, 1970). Furthermore, it has important implications for micro-evolutionary processes (Igic et al. 2008) and macro-evolutionary patterns of clade diversification (Takebayashi and Morell 2001; Goldberg et al. 2010; Ferrer and Good 2012; Chapter 2, this thesis). Much of the evolutionary significance of the loss of self-incompatibility relates to the notion that its loss is the prerequisite for the transition from allogamous (outcrossing) to predominantly autogamous (selfing)

mating (Stebbins 1970; Barrett 2002; Busch and Schoen 2008; Wright et al. 2008; Karron et al. 2012; Raduski et al. 2012). While self-incompatible flowers are necessarily outcrossing, self-compatible flowers can either outcross, self or have an intermediate selfing rate, but high rather than low selfing is more common for self-compatible taxa (Raduski et al. 2012). Commonly, transitions toward increased selfing after the loss of self-incompatibility are associated with a suite of changes in morphological and reproductive floral characters (Darwin 1876, Ornduff 1969, Stebbins 1970), including a decreased floral display, a reduced pollen-to-ovule-number ratio, a smaller distance between male and female organs within flowers (i.e. less herkogamy) and a general reduction in floral size, collectively termed the "selfing syndrome" (see Table 1 in Ornduff 1969; Cruden 1977; Ritland and Ritland 1989; Goodwillie et al. 2010; Sicard and Lenhard 2011).

The selfing syndrome is considered a common phenomenon; transitions from outcrossing to increased selfing are thought to be "in most cases" (Sicard and Lenhard 2011) if not "almost universally" (Foxe et al. 2009) associated with the selfing syndrome. Stebbins (1970, p. 310) stated in an early discussion that "in all self-fertilizers, flower size diminishes below that found in their cross-fertilizing ancestors", suggesting that evolution toward a selfing syndrome upon the loss of self-incompatibility is a unidirectional, deterministic evolutionary trend. Yet, most of our understanding of the evolution of floral traits after the loss of self-incompatibility stems from explicit analyses on a few selected taxa (e.g. *Capsella*, Slotte et al. 2010; *Eichhornia*, Vallejo-Marín and Barrett 2009; *Leavenworthia*; Busch and Urban 2011; *Mimulus*, Ritland and Ritland 1989), or from informal interpretation of data on large numbers of species (e.g. Darwin 1876; Ornduff 1969; Stebbins 1970). Few comparative studies involving a larger number of species in an explicit phylogenetic framework have been conducted (but see Goodwillie et al. 2010 for an angiosperm-wide analysis of floral display in inflorescences and selfing rates). Specifically, although multiple independent losses of self-incompatibility are documented in several clades (e.g. *Linanthus* section *Leptosiphon* (Polemoniaceae), Goodwillie 1999; Solanaceae, Goldberg et al. 2010; Triticeae (Poaceae), Escobar et al. 2010), it is unclear whether, or to what extent, replicate transitions in different species within a clade lead to similar evolutionary trajectories. Are the floral displays of self-compatible species always smaller than their self-incompatible relatives, as Stebbins (1970) suggested? Do individual floral traits respond differently to increased selfing? Do different floral traits evolve synchronously or asynchronously to the loss of self-incompatibility? These questions were identified as "unsolved mysteries in the transition to self-fertilization" (Karron et al. 2012) and are addressed in the current study.

Several reasons that are not mutually exclusive have been proposed for the correlation between decreased floral size and increased selfing rates (Sicard and Lenhard 2011). First, small floral size may facilitate autonomous selfing and be directly targeted by selection, for instance when selfing provides reproductive assurance under mate- or pollinator-limited conditions (Eckert et al. 2006). Second, if reproductive fitness is decoupled from the attractiveness of floral display for pollinators, as is the case in strict selfers, theory predicts that resources would not be invested in large flowers, but rather in increased reproduction (e.g. ovule production; Brunet 1992). Third, the selfing syndrome may be a pleiotropic effect of selection for small flowers driven by selection for the avoidance of herbivory (Eckert et al. 2006) or by selection for fast maturation in marginal habitats (Guerrant 1989; Aarssen

2000). These reasons suggest that after a transition toward increased selfing, floral size is under selection to progressively diminish in a range of scenarios.

Despite the broad acceptance of the selfing syndrome, the loss of self-compatibility does not necessarily result in small floral size. In fact, showy flowers with highly specialized pollination systems are often self-compatible and can have high selfing rates, in contrast with the prediction of the selfing syndrome (reviewed by Fenster and Martén-Rodríguez 2007). The occurrence of high selfing rates in showy, specialized flowers contradicts the interpretation that showiness is the product of selection for high maternal outcrossing rates (Fenster and Martén-Rodríguez 2007). This conflict may be explained by the idea that showy, specialized flowers, relying on a small subset of the potential pollinator community, are inherently prone to reproductive failure, and thus selfing may assure reproduction when outcrossing fails. Therefore, Fenster and Martén-Rodríguez (2007) concluded that a showy floral display and pollinator specialization may evolve due to selective forces independent of those operating on the selfing rate. The notion that floral display may evolve independently from the selfing rate (Fesnter and Marten-Rodriguez 2007) implies that the loss of self-incompatibility does not necessarily lead to small flowers, as predicted by the selfing-syndrome theory. It is thus topical to ask whether floral traits respond to the loss of self-incompatibility consistently across different species.

In this study, we assess the extent to which the loss of self-incompatibility and the associated possibility of increased selfing results in a unidirectional, deterministic evolutionary trajectory towards smaller floral size, as predicting by the selfing syndrome, using the primroses as our study system. This group of ca. 550 species (i.e. the clade "/Primula" sensu Mast et al. 2001, that is, *Primula* and nested genera, Primulaceae; Richards 2003; see also Chapter 2, this thesis) is a classic model for the evolution of selfing, discussed in the seminal works of Ornduff (1969) and Stebbins (1970) as an example of a clade demonstrating the repeated loss of self-incompatibility and associated origin of selfing lineages, in the form of transitions from heterostyly to homostyly. Heterostyly is a form of heteromorphic self-incompatibility in which populations consist of two (distyly) or three (tristyly) genetic morphs that differ in their reciprocal placement of sexual organs and in their mating type, so that only crosses between morphs show full fertility (reviewed by Ernst 1962; Ganders 1979; Barrett 1992; Wedderburn and Richards 1992; Barrett and Shore 2008; Cohen 2010; Naiki 2012). Homostylous species have only one floral morph (i.e., are monomorphic), are self-compatible and hence, self-fertilization is possible. Detailed phylogenetic studies concluded that the crown node of the clade /Primula was heterostylous and indicated several, deeply nested losses of heterostyly within the clade (Mast et al. 2006, De Vos et al. 2012). Similar patterns occur in many of the ca. 28 plant families with heterostyly, with homostylous species evolving multiple times independently from heterostylous ancestors (besides in *Primula* e.g. in *Amsinckia*, Boraginaceae, Schoen et al. 1997; *Narcissus*, Amaryllidaceae, Graham and Barrett 2004; *Nymphoides*, Menyanthaceae, Tipperey and Les 2011; Pontederiaceae, Kohn et al. 1996; *Turnera*, Turneraceae, Truyens et al. 2005). The recurrent transition from heterostyly to homostyly is an important model for the evolution of selfing in angiosperms (reviewed by Barrett 2003), making it an ideal system to evaluate the selfing syndrome from a quantitative, comparative perspective.

Here, we analyze a large data set of multiple, quantitative floral traits in /Primula in a phylogenetic framework by using a combination of recently developed methods that employ explicit

models of quantitative trait evolution and account for both evolutionary relationships and intraspecific variation. We ask the following questions: Do heterostylous and homostylous species differ in (i) overall floral morphology and (ii) individual floral traits? (iii) How does the evolutionary trajectory (e.g., the inferred selective optimum) of each floral trait change upon the loss of self-incompatibility? By answering these questions, our study contributes to an understanding of the phenotypic consequences of the loss of self-incompatibility, one of the most important evolutionary transitions in flowering plant evolution.

Methods

Phylogeny

In this study, we used the 265-taxon, time-calibrated phylogeny of Primulaceae s.str. (Primulaceae subfamily Primuloideae, sensu Angiosperm Phylogeny Group 2009) estimated by De Vos et al. (Chapter 2, this thesis). Taxon sampling was designed to cover the morphological variation in the family, by including species from all genera and all sections, representing ca 35% of extant diversity. Phylogenetic relationships were inferred from four chloroplast markers using the uncorrelated lognormal relaxed clock method in BEAST v.1.6.2 (Drummond and Rambaut 2007). We calculated the maximum clade credibility (MCC) tree with median node heights for the /Primula clade (*Primula* and the nested genera *Dionysia*, *Cortusa* and *Dodecatheon*) from 1000 samples from the posterior distribution of phylogeny estimates for Primulaceae of De Vos et al., after pruning all species outside of /Primula from each sample. Subsequently, we removed branches from the MCC tree representing species for which no quantitative floral data was available (see below).

Morphological data

For the designation of a species as heterostylous we relied on the accounts in "Flora of China" (Hu and Kelso 1996), Richards' (2003) comprehensive monograph of *Primula*, Grey-Wilson's (1989) account of *Dionysia*, and the extensive review by Ernst (1962). For analyses that did not account for intra-specific variation, we followed Ernst (1962) in scoring a species' predominant breeding for species with multiple breeding systems reported.

Quantitative floral measurements were assembled from three sources. First, detailed data on the floral morphology of *Primula* species were meticulously reported in the series "Stammesgeschichtliche Untersuchungen zum Heterostyly-Problem" by Ernst (1938, 1949, 1953, 1956, 1959, 1961, 1962) for a total of approximately 835 pages. These data, consisting of ten measurements on each of 2680 flowers representing 138 currently accepted species, were digitized using Optical Character Recognition software (Readiris Pro v.11, I.R.I.S. Group S.A., Louvain-la-Neuve, Belgium) on high-resolution scans, manually corrected, and proof-read twice. We followed the most recent comprehensive monograph of *Primula* for species synonymy (Richards 2003). Secondly, we extracted ranges and means of the respective floral traits from "Flora of China" (Hu and Kelso 1996) for the ca. 300 Chinese *Primula* species. The ranges listed in this treatment are differentiated between heterostyly and homostyly and typically stem from observations on multiple herbarium sheets per species in multiple herbaria (pers. comm., S. Kelso), ensuring that intraspecific variation is adequately captured. Finally,

for the species of *Dionysia*, we used the information provided in the monograph of Grey-Wilson (1989). We did not include measurements from species of the nested genus *Dodecatheon,* because their aberrant floral structure (Mast et al. 2004) impedes meaningful quantitative comparisons of the size of floral organs to other species in the clade.

Among the available floral measurements, we selected four floral traits that are thought to influence a plant's mating system: the distance from the base of the flower to (a) the apex of the calyx (i.e., calyx length) and (b) to the mouth of the corolla-tube (i.e., tube length), (c) the diameter the corolla limb (i.e., corolla diameter), and (d) the absolute distance between the top of the male (anthers) and female (stigmas) organs within flowers (i.e., herkogamy). We included the compound trait herkogamy rather than the position of anthers and stigmas separately, because it is problematic to compare anther and stigma positions of species with and without heterostyly. Moreover, herkogamy has been shown to affect the genetic selfing rate (e.g., Herlihy and Eckert 2007) and the probability of autonomous self-fertilization (De Vos et al. 2012) and is therefore a more meaningful to compare between heterostylous and homostylous species than the absolute position of sexual organs. Some floral characters for which data was available, for ex., the length of the calyx teeth or the degree of incision of the corolla lobes, were excluded from further analyses, because we expected strong correlations with the traits we included. For analyses not accounting for intra-specific variation, we calculated the means of the four traits listed above in all the 126 species of the phylogeny by De Vos et al. (Chapter 2, this thesis) for which data were available.

Ancestral state inference

To assess the number of independent losses of heterostyly captured by the taxon sampling of the current study, we inferred the presence/absence of heterostyly at ancestral nodes in a likelihood framework, using the function ace in the R-package ape (Paradis et al. 2004). We calculated the likelihood of the data under the alternative models of equal rates of gain and loss (the SYM model) and different rates of gain and loss (the ARD model) using each of 1000 trees of the posterior distribution of trees from which the MCC tree was calculated. We assessed model fit based on the distribution of AIC scores and calculated the likelihoods associated with presence/absence of heterostyly at all ancestral nodes in the MCC tree.

Floral differentiation

To quantify the extent of phylogenetic signal in all four traits, we used Pagel's (1999) lambda, a scaling parameter of the off-diagonal elements of the phylogenetic variance-covariance matrix, as implemented in the R-package geiger (Harmon et al. 2009), because this measure performed comparatively well among a set of estimators of phylogenetic signal (Münkemüller et al. 2012). We determined if lambda was significantly different from both zero and one using likelihood-ratio tests.

To summarize quantitative variation and covariation of all floral traits among species with and without heterostyly, we performed a phylogenetic principal component analysis using the function phyl.pca in the R-package phytools (Revell 2012) on mean values per species and trait (Revell 2009). We employed the appropriate scaling factor for branch lengths determined by the test for phylogenetic signal.

To test whether individual floral traits differ between species with and without heterostyly, we used four generalized linear mixed models, one for each floral trait, implemented in the R-package MCMCglmm (Hadfield 2010), which accounts for both intraspecific variation and phylogenetic relatedness of species. We used "presence of heterostyly" as predictor variable, fitted a univariate normal response to the data of each floral trait, and included phylogeny and intraspecific variation as random variables. Models were run for 2,500,000 iterations with a burnin of 1,000,000 iterations and a thinning interval of 1000 iterations. We adjusted the standard, weak priors to facilitate convergence by splitting the observed total variance in our response variables in equal parts between the random (phylogenetic and intra-specific) and the residual variance components. We assessed the significance of the predictor's effect by determining if the 95% credible interval (95% CI) of the effect size (i.e. the difference between intercepts) included zero.

Models of trait evolution

To test whether the evolutionary trajectories (see below) of floral traits differ between heterostylous and homostylous species, we fitted a series of likelihood models for continuous characters and compared the estimated parameters among the most likely candidate models. To this end, we modeled quantitative trait evolution as a Ornstein–Uhlenbeck (OU) stochastic process, which describes a combination of random drift (termed Brownian Motion; BM) and a deterministic, selective "pull" toward an optimal value, termed θ (Hansen 1997; Butler and King, 2004; Beaulieu et al. 2012). The evolution of the trait toward θ is governed by a constant describing the strength of selection, termed α, and a constant that measures the intensity of drift-like random fluctuations in the evolutionary process, termed σ^2. When α=0, the model collapses to BM (hereafter BM_1); when α>0, the model is termed OU_1, where the subscript "1" refers to the presence of a single, global optimum θ. Although these models employ terms similar to those used for micro-evolutionary processes (e.g. drift, selection), they actually describe the pattern of evolutionary change, i.e. the evolutionary trajectory (Beaulieu et al. 2012). To avoid confusion, we make an explicit distinction between genetic drift, which is a population-genetic process, and macro-evolutionary drift, described by the model-parameter σ^2, throughout the paper.

Recently, these models of quantitative trait evolution have been generalized to incorporate multiple values for θ, α, and/or σ^2 that can be associated with the evolution of discrete character-states along the phylogeny (Butler and King 2004, O'Meara 2006, Beaulieu et al. 2012). The mapped history of a character, heterostyly in the current context, divides the phylogeny in heterostylous and homostylous partitions; θ, α, and/or σ^2 are then fitted to the quantitative data with global or partition-specific values. By comparing support for models that either have single or multiple values for θ, α, and/or σ^2, we can thus determine which aspects of the evolutionary trajectory change with the loss of heterostyly (Table 1).

We considered five models with multiple θ, α and/or σ^2. The BM_S model includes one global, optimal trait value θ, but the intensity of the stochastic fluctuations, σ^2, can differ along the phylogeny as determined by the presence or absence of a character-state (O'Meara et al., 2006). The OU_M model, with two θ but one α and one σ^2, describes the situation where a floral trait may evolve toward different optimal values, for instance indicated by a smaller θ for homostylous than heterostylous species, while

the rate of evolution towards these optima is the same (Butler and King 2004). Beaulieu et al. (2012) recently implemented expanded OU_M-models in which, besides θ, also α or σ^2 varies with the character history (i.e. OU_{MA} and OU_{MV}, respectively). In the most general case, θ, α and σ^2 are each estimated separately for heterostylous and homostylous tree partitions (the OU_{MVA} model; Beaulieu et al. 2012).

Table 1. Models of quantitative-trait evolution relevant to this study with their parameters and biological interpretation, indicating for each model whether the optimal trait value, θ, the intensity of random fluctuations in the evolutionary trajectory, σ^2, and the selective "pull" toward the optimal value, α, is modeled with one global parameter or with two parameters that are heterostyly- and homostyly-specific.

Model	Parameters			Interpretation for quantitative trait evolution
	Theta	***Sigma²***	***Alpha***	
BM_1	Global	Global	-	Evolution is random and not affected by the loss of heterostyly
BM_S	Global	State-specific	-	Evolution is random but the loss of heterostyly affects the rate of change
OU_1	Global	Global	Global	Evolution is directed toward an optimum value without being affected by the loss of heterostyly
OU_M	State-specific	Global	Global	The loss of heterostyly is associated with a shift toward a different optimal value
OU_{MA}	State-specific	Global	State-specific	The loss of heterostyly is associated with a shift toward a different optimal value that exerts a different selective pull
OU_{MV}	State-specific	State-specific	Global	The loss of heterostyly is associates with shifts toward a different optimal value and in the rate of random change

Note that the information in the data was insufficient to fit OU_{MVA} models (containing state-specific θ, α and σ^2), hence, these were not further considered (see text).

Implementation

To compare how well these seven models (two models with global and five models with multiple values for θ, α and/or σ^2, respectively) fit the floral-trait data of heterostylous and homostylous species, we first assigned each species to either breeding system. We then used stochastic character mapping (Huelsenbeck et al. 2003) implemented in the R-package phytools (Revell 2012) to sample 100 possible histories of the loss of heterostyly given the maximum likelihood estimate of the rate of change in presence of heterostyly. We followed Mast et al. (2006) in using the “equal-rates” transition model (i.e. SYM) for the evolution of heterostyly in /Primula, but also tested the “all-rates-different” transition model (i.e. ARD). We chose to use stochastic maps, rather than the maximum likelihood estimate, to allow for incorporation of uncertainty in the evolutionary history of heterostyly in the estimation of differences between the evolutionary trajectories of floral traits of heterostylous and homostylous species, a strategy that was found to be useful in other studies (e.g. Price et al. 2012).

Models were fitted using the R-package OUwie (Beaulieu et al. 2012). To facilitate model fitting, we divided all trait values by ten and adjusted the initial values of the likelihood search, trying values of 0.01, 0.3, or 1.0. Nevertheless, for most mapped histories, it was impossible to fit the most complex model, OU_{MVA}, to the data. Inspection of the eigendecomposition of the Hessian matrix and examination of the eigenvectors, as recommended by Beaulieu et al. (2012), revealed that problematic inference was usually related to difficulties in estimating α jointly with σ^2 from the data. Therefore,

OU_{MVA} models were considered too complex for the information contained in the data and abandoned (Beaulieu et al. 2012). We also excluded mapped histories for which the maximum likelihood could not be determined reliably in all models as indicated by negative eigenvalues of the Hessian (Beaulieu et al. 2012). Model fit was determined using AICc weights calculated from ΔAICc scores (Burnham and Anderson 2002). AICc weights can be interpreted as the probability that a model is the best one among the candidate models. We considered models with AICc weights < 0.05 to be not supported by the data and all models with AICc weight > 0.05 to be plausible. As advised by Beaulieu et al. (2012), we interpret results by comparing differences in parameter estimates between heterostylous and homostylous species among the set of plausible models for the evolution of each trait.

Expectations

The selfing-syndrome theory predicts that flower size should be lower in homostylous than heterostylous species. Therefore, models with two optima, θ (OU_M, OU_{MA}, and OU_{MV}) are expected to receive higher AICc weights than models with one θ (BM_1, BM_S, OU_1) and the inferred θ should be smaller for homostylous species. Secondly, selfing is generally expected to lead to lower effective population sizes, which in turn implies that genetic drift becomes more important in the evolutionary process (Lloyd, 1980; Hamrick and Godt, 1996). Thus, floral trait evolution is expected to be more stochastic in homostylous than in heterostylous species, because homostylous species are likely to have higher selfing rates than heterostylous species. Therefore, we predict high AICc weight for the model that allows for two σ^2 (OU_{MV}), with higher σ^2 for floral traits of homostylous species. Finally, the self-compatible flowers of homostylous species rely less on providing an adequate fit to their pollinator(s) for reproduction than obligately outcrossing, heterostylous species. Consequently, homostylous species are expected to be less affected by selective constraints imposed on floral traits by pollinators than heterostylous species. Instead, homostylous species are expected to experience stronger selection for low herkogamy to facilitate self-fertilization. Therefore, we predict that the model with two α (OU_{MA}) receives high AICc weight for all traits. In addition, we predict that homostylous species have a higher α for herkogamy, but lower α for other traits, compared to heterostylous species.

Results

Ancestral state inference

The likelihood of the data given an asymmetrical model of character evolution (ARD) was higher than under the less complex, symmetrical model (SYM), as reflected in lower AIC scores (ARD: 85.47±0.05; mean AICc among all trees ±1 SE; SYM: 99.59±0.07). However, the ARD model inferred a 9-fold higher rate of gain than loss of heterostyly (q_{01}: 0.425±0.004; q_{10}: 0.047±0.001), which seems highly unrealistic and could be an artifact. The inference of a high forward rate q_{01} appears to be driven by the presence of 2-3 implied re-gains of heterostyly over short branches (*Primula farinosa, P. aurantiaca,* and perhaps *P. pulverulenta*), but the particular topological relationships are not well supported and partially in conflict with detailed studies at the sectional level (Guggisberg et al. 2006, 2009). After repeating the analysis without these three species, the ARD model was indeed no longer supported over the SYM model (not shown). Figure 1 illustrates the

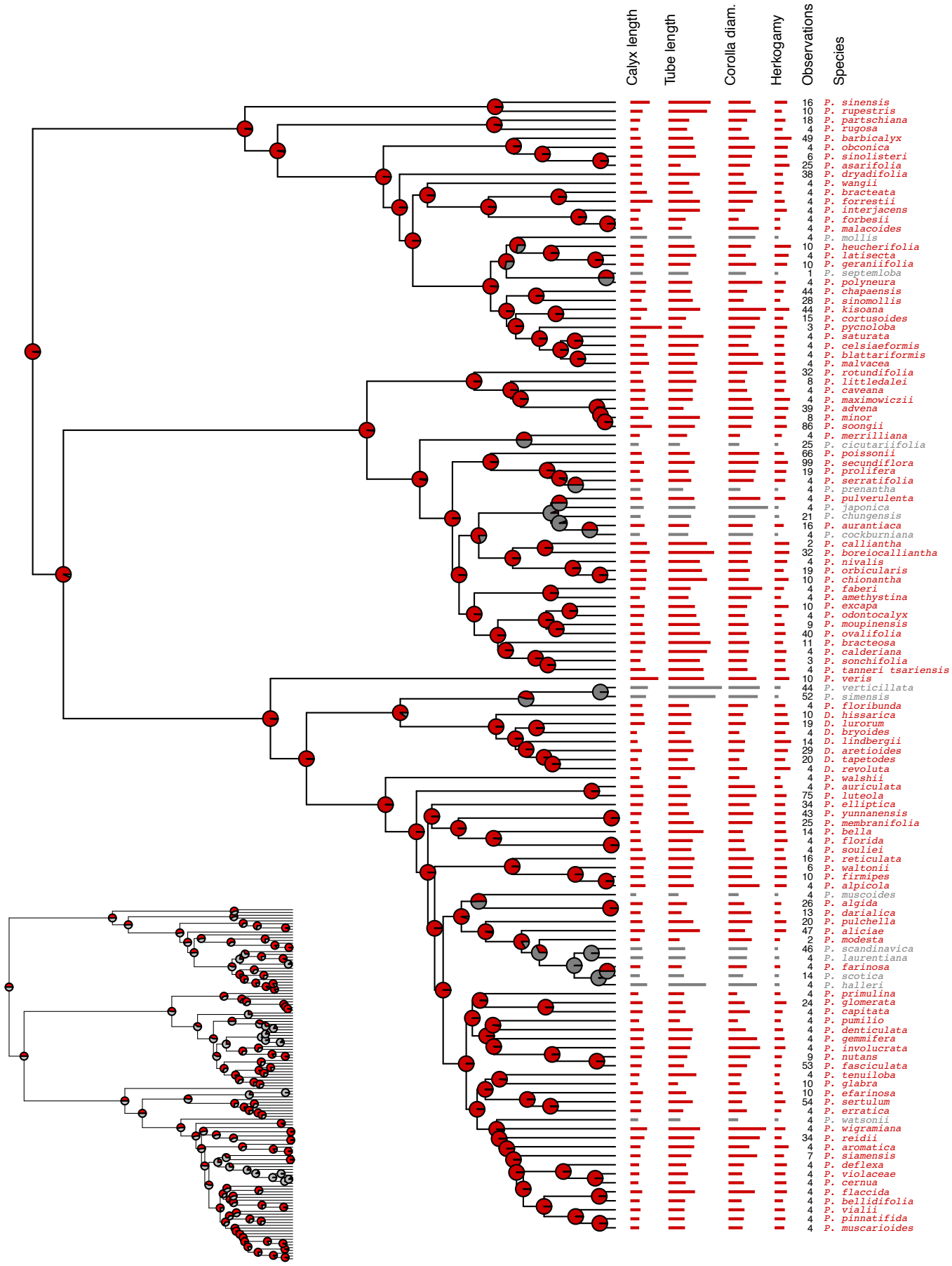
Calyx length
Tube length
Corolla diam.
Herkogamy
Observations
Species
16 P. sinensis
10 P. rupestris
18 P. partschiana
4 P. rugosa
49 P. barbicalyx
4 P. obconica
6 P. sinolisteri
25 P. asarifolia
38 P. dryadifolia
4 P. wangii
4 P. bracteata
4 P. forrestii
4 P. interjacens
4 P. forbesii
4 P. malacoides
4 P. mollis
10 P. heucherifolia
4 P. latisecta
10 P. geraniifolia
1 P. septemloba
4 P. polyneura
44 P. chapaensis
28 P. sinomollis
44 P. kisoana
15 P. cortusoides
3 P. pycnoloba
4 P. saturata
4 P. celsiaeformis
4 P. blattariformis
4 P. malvacea
32 P. rotundifolia
8 P. littledalei
4 P. caveana
4 P. maximowiczii
39 P. advena
8 P. minor
86 P. soongii
4 P. merrilliana
25 P. cicutariifolia
66 P. poissonii
99 P. secundiflora
19 P. prolifera
4 P. serratifolia
4 P. prenantha
4 P. pulverulenta
4 P. japonica
21 P. chungensis
16 P. aurantiaca
4 P. cockburniana
2 P. calliantha
32 P. boreiocalliantha
4 P. nivalis
19 P. orbicularis
10 P. chionantha
4 P. faberi
4 P. amethystina
10 P. excapa
4 P. odontocalyx
9 P. moupinensis
40 P. ovalifolia
11 P. bracteosa
4 P. calderiana
3 P. sonchifolia
4 P. tanneri tsariensis
10 P. veris
44 P. verticillata
52 P. simensis
4 P. floribunda
10 D. hissarica
19 D. lurorum
4 D. bryoides
14 D. lindbergii
29 D. aretioides
20 D. tapetodes
4 D. revoluta
4 P. walshii
4 P. auriculata
75 P. luteola
34 P. elliptica
43 P. yunnanensis
25 P. membranifolia
14 P. bella
4 P. florida
4 P. souliei
16 P. reticulata
6 P. waltonii
10 P. firmipes
4 P. alpicola
4 P. muscoides
26 P. algida
13 P. darialica
20 P. pulchella
47 P. aliciae
2 P. modesta
46 P. scandinavica
4 P. laurentiana
4 P. farinosa
14 P. scotica
4 P. halleri
4 P. primulina
24 P. glomerata
4 P. capitata
4 P. pumilio
4 P. denticulata
4 P. gemmifera
4 P. involucrata
9 P. nutans
53 P. fasciculata
4 P. tenuiloba
10 P. glabra
10 P. efarinosa
54 P. sertulum
4 P. erratica
4 P. watsonii
4 P. wigramiana
34 P. reidii
4 P. aromatica
7 P. siamensis
4 P. deflexa
4 P. violaceae
4 P. cernua
4 P. flaccida
4 P. bellidifolia
4 P. vialii
4 P. pinnatifida
4 P. muscarioides

Fig. 1 (previous page). Maximum clade credibility chronogram and characters states of the clade /Primula. Main figure: pie charts at internal nodes indicate the proportion of likelihood associated with the ancestral state being heterostylous (in red) and homostylous (in grey), based on the SYM-model, in which rates of losses and gains of heterostyly are constraint to be equal. Bars to the right of the tree are drawn with length proportional to the mean value in mm per species of the four analyzed floral traits (left to right: calyx length, corolla-tube length, corolla-limb diameter, and herkogamy), where red and grey bars are used for heterostylous and homostylous species, respectively. The column Observations indicates the number of observations that were available to calculate species means and account for intraspecific variation in the MCMCglmm analyses. Inset figure: phylogeny with pie charts indicating ancestral states as for the main figure, but based on the ARD-model, in which rates of losses and gains of heterostyly are estimated separately. Despite the apparent difference between the ARD and SYM ancestral state reconstructions, the results of downstream analyses were qualitatively the same.

ancestral states at internal nodes of the MCC tree as the proportion of likelihood associated with presence/absence of heterostyly under the SYM model. The deeper nodes are significantly more likely to be heterostylous than homostylous; the distribution of states at the tips imply that our phylogenetic sampling captures nine independent losses of heterostyly, indicating that our data provides a good model for the repeated loss of self-incompatibility.

Floral differentiation

All quantitative floral traits showed significant phylogenetic signal (corolla-limb diameter: $p=0.041$; other traits $p<0.001$). Values of Pagel's lambda were 0.826 (calyx length), 0.772 (tube length), 0.525 (corolla-limb diameter), and 0.778 (herkogamy). This justifies analysis and interpretation of the floral data in a phylogenetic context.

The phylogenetic principle component analysis produced four axes (PCs) that explained 50.4%, 20.8%, 15.9% and 12.9% of variance, respectively. PC 1 was negatively correlated with all traits (factor loadings between -0.59 and -0.71), whereas PC 2 was correlated strongly and positively with herkogamy (factor loading 0.72) and negatively with corolla-limb diameter (factor loading -0.49). The scatterplot diagram of PCs 1 and 2 showed that the PCA scores of species with and without heterostyly largely overlapped on axis 1, but species without heterostyly had generally lower scores on PC 2 (Fig. 2).

The MCMCglmm analyses, which accounted for intraspecific variation and phylogenetic relatedness of species, indicated that all investigated floral traits were significantly different between homostylous and heterostylous species (Fig. 3). Although the 95% credible intervals (CIs) of posterior means of heterostylous and homostylous species overlapped considerably (Fig. 4), the CIs of the effect size of homostyly (i.e. the relative difference in size of traits in homostylous compared to heterostylous species) did not include zero for any trait (Fig. 3). The directionality of change differed among traits, as the sign of the effect sizes differed among traits (Fig 3). In contrast with the expectations under the selfing syndrome, homostylous species tended to have longer corolla tubes and calyces than related heterostylous species (95% CIs of the effect size 0.08 to 1.07 and 0.62 to 1.07, respectively; Figs 3, 4AB). Congruent with the selfing syndrome, corolla-limb diameter and herkogamy tended to be smaller in homostylous species (95% CIs of the effect size -1.79 to -0.39 and -4.24 to -4.51, respectively; Figs 3, 4CD).

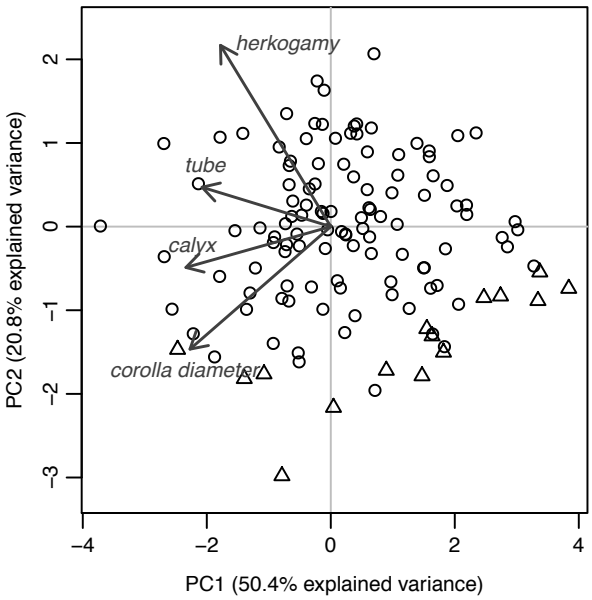

Figure 2. Scatterplot diagram of phylogenetic principal component analysis on four floral traits (calyx length, corolla-tube length, corolla-limb diameter, and herkogamy) for the first two principal components (PC1, PC2). Triangles and circles represent homostylous and heterostylous species, respectively. Arrows indicate factor loadings on PC1 and PC2. The two principal components together explain 71.18% of total variance among species.

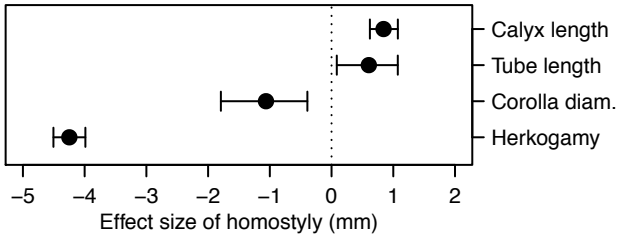

Figure 3. Posterior estimates of the effect size of homostyly on calyx length, corolla-tube length, corolla-limb diameter and herkogamy. Dots represent the mean of the posterior estimate with 95% credible intervals (CIs) represented by horizontal bars. Because the 95% CIs do not overlap with zero, all traits significantly differ between heterostylous and homostylous species: calyx and corolla tube are longer in homostylous species (positive effect size), whereas corolla-limb diameter and herkogamy are larger in heterostylous species (negative effect size).

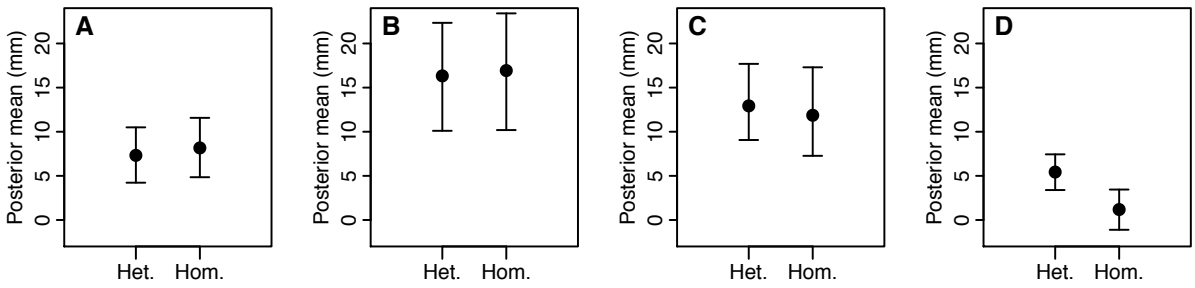

Figure 4. Posterior means of traits of heterostylous (Het.) and homostylous (Hom.) species in (A) calyx length, (B) corolla-tube length, (C) corolla-limb diameter, and (D) herkogamy inferred from the MCMCglmm analyses. Dots represent the overall meta-analytical posterior means with 95% credible intervals (CIs) represented by the vertical bars. Although the overall posterior means overlap strongly between heterostylous and homostylous species, the effect due to homostyly is significant for all traits (see also Fig. 3).

Models of trait evolution

The results of the model-fitting for six models of quantitative trait evolution are summarized in Table 2 as means across 100 stochastic maps with standard error and associated AICc weights. Results including models not supported by the data are given in Table S1 (Supplementary Information). We only report results based on stochastic maps simulated under the SYM model, as results based on the ARD model stochastic maps were qualitatively congruent. Quantitatively, differences between heterostylous and homostylous species were less pronounced under the ARD approach (Table S1).

The best model for calyx length was OU_M (AICc weight 0.42), where θ was slightly lower for homostylous than for heterostylous species (0.53 and 0.69, respectively), congruent with the MCMCglmm results and predictions of the selfing syndrome. Although OU_{MA} and OU_{MV} also received considerable AICc weight (0.16 and 0.17, respectively), estimates of σ^2 and α were similar between heterostylous and homostylous species, indicating that if there are differences at all, they are small.

The best model for corolla tube length was OU_{MV} (AICc weight 0.67). In contrast with Congruent with our predictions, θ was smaller for homostylous species then heterostylous species (1.28 and 1.52, respectively) and σ^2 was much higher in homostylous species (0.60 versus 0.16 in heterostylous species), which fits our prediction that macro-evolurionary drift is more important in homostylous species. The θ optima for both calyx and corolla tube lengths inferred by model fitting appeared to be incongruent with posterior estimates from MCMCglmm analyses, which inferred slightly higher mean trait values to homostylous species.

The OUMA model received only half as much support (AICc weight 0.33) as the OUMV model and indicated a higher α for homostylous than heterostylous species. This would be contrary to our expectations.

The diameter of the corolla limb was also best modeled under OU_{MV} (AICc weight 0.39). Interestingly, θ was nearly the same between heterostylous and homostylous species (1.28 and 1.27, respectively), but, congruent with the results of corolla tube length, σ^2 was again higher in homostylous species (3.70 versus 2.04 in heterostylous species), suggesting that higher levels of stochasticity affected the evolution of both traits after the loss of heterostyly. OU_M and OU_1 also received some support (AICc weight both 0.27), as did OU_{MA} (AICc weight 0.07), but both α were nearly the same under the latter model, making it effectively identical to the OU_M model.

For herkogamy, the OU_{MV} model received AICc weight of 1.00, indicating that it is the only plausible model. Congruent with the selfing syndrome, homostylous species had lower θ than heterostylous species (0.10 and 0.50, respectively). In contrast to the other traits for which OU_{MV} was the best model, σ^2 was lower in homostylous species (0.56 versus 0.09 in homostylous species).

Table 2. Model fit and estimated parameters of plausible models (AICc weight > 0.05) for the four floral traits, indicating corrected AIC score (AICc), AICc weight, and the estimated values of the parameters θ (theta; optimum in cm), α (alpha, selective pull) and σ^2 (sigma^2; rate of random drift). When models contain a single, global parameter, estimates are italicized and printed in the center of the column; estimates for heterostyly- and homostyly-specific parameters are reported in their respective columns.

Trait	Model	AICc	AICc weight	*Theta*		*Sigma*^{2}		*Alpha*	
				Heterostyly-specific	Homostyly-specific	Heterostyly-specific	Homostyly-specific	Heterostyly-specific	Homostyly-specific
Calyx length	OU_1	12.642±0.000	0.247±0.006	*0.684±0.000*		*0.958±0.000*		*6.351±0.000*	
	OU_M	11.578±0.056	0.424±0.005	0.686±0.003	0.525±0.005	*0.047±0.000*		*0.313±0.000*	
	OU_{MA}	14.138±0.478	0.157±0.003	0.692±0.002	0.521±0.005	*0.045±0.000*		0.301±0.005	0.305±0.005
	OU_{MV}	13.427±0.091	0.172±0.007	0.686±0.004	0.522±0.005	0.046±0.001	0.055±0.001	*0.308±0.001*	
Corolla-tube length	OU_{MA}	201.951±1.555	0.321±0.025	1.566±0.008	1.333±0.009	*0.127±0.004*		0.179±0.014	0.219±0.013
	OU_{MV}	191.999±0.162	0.666±0.025	1.520±0.006	1.280±0.009	0.163±0.008	0.604±0.010	*0.268±0.000*	
Corolla-limb diameter	OU_1	173.267±0.000	0.269±0.005	*1.278±0.000*		*60.242±0.000*		*136.543±0.000*	
	OU_M	173.265±0.000	0.270±0.005	1.277±0.000	1.281±0.000	*2.944±0.002*		*6.674±0.004*	
	OU_{MA}	189.454±2.787	0.068±0.005	1.276±0.001	1.131±0.021	*0.359±0.019*		0.787±0.053	0.809±0.053
	OU_{MV}	172.511±0.080	0.393±0.008	1.278±0.000	1.274±0.002	2.041±0.073	3.692±0.126	*4.964±0.164*	
Herkogamy	OU_{MV}	-81.731±0.201	0.996±0.001	0.502±0.013	0.100±0.013	0.564±0.036	0.088±0.023	*7.845±0.428*	

Parameter estimates are reported as mean ± standard error across 100 stochastic maps generated using the SYM model for the evolution of heterostyly. See table S1 in the Supporting information for results including models not supported by the data (AICc weight <0.05) and for results based on stochastic maps generated under the ARD model.

Discussion

The commonly expected effect of the loss of self-incompatibility on the evolutionary trajectories of floral traits is a unidirectional, deterministic trend toward small floral size and shifted resource allocation (i.e., the selfing syndrome), mainly because species that self have smaller returns from investment in traits that attract pollinators (Sicard and Lenhard 2011). Our results are partially congruent with evolution toward smaller floral traits upon the loss of self-incompatibility (i.e. loss of heterostyly), as self-compatible species (i.e. homostyles) have smaller selective optima, θ, for all traits in the evolutionary models inferred to be most plausible (Table 2). However, our analyses also suggest a more complex, versatile evolutionary fate of self-compatible lineages. First, we find that homostylous species span a similar range of variation in overall floral morphology as heterostylous species, with the exception of herkogamy (compare PC 1 and PC 2 and their factor loadings, Fig. 2). Second, although our Bayesian glmm and evolutionary-model fitting analyses indicated that all floral traits differ between heterostylous and homostylous species (effect sizes do not include zero, Fig 3; strongest support for evolutionary models that differentiate heterostylous and homostylous species, Table 2), the posterior means of corolla-tube and calyx-length traits were significantly higher in homostyles, whereas corolla-limb diameter and herkogamy were significantly larger in heterostylous species (Fig. 3), indicating contrasting effects of the loss of heterostyly on different floral traits. Third, the best supported models for corolla-limb diameter and corolla-tube length are characterized by strongly different degrees of stochastic fluctuations during evolution, σ^2, with much higher stochasticity affecting trait evolution in homostyles (Tables 1, 2). Taken together, the variability and direction of change in the floral traits of homostylous species detected by our analyses contrast with the traditional paradigm of the selfing syndrome and with Stebbins' (1970) influential remark that selfing lineages *always* have smaller flowers than their outcrossing relatives.

The transition from heterostyly to homostyly is a classic system to investigate the genetic, ecological, and population biological contexts for the evolution of selfing (reviewed e.g. by Ernst 1955; Lewis and Jones 1992; Barrett 1992; Barrett 2003; Barrett & Shore 2008; Naiki 2012). Field experiments revealed a high capacity for self-fertilization in several homostylous primroses (e.g. Washitani et al. 1994; Chen 2009; Carlson et al. 2010; De Vos et al. 2012), although genetic estimates of the actual selfing rate are rarely available (Piper et al. 1984). Our analyses indicate that homostylous species have strongly reduced herkogamy (Figs 3, 4) and a lower selective optimum, θ, for herkogamy, which was only slightly larger than zero (Table 2). Since herkogamy typically correlates negatively with the degree of auto-fertility (in e.g. *Primula*, De Vos et al. 2012; *Aquilegia*, Herlihy and Eckert 2007) the low - though non-zero - average herkogamy of homostylous species suggests that these species do not exclusively self (De Vos et al. 2012), but generally have selfing rates distinctly higher than self-incompatible heterostylous species. The transition from heterostyly to homostyly and the associated loss of self-incompatibility is thus a well-suited system for testing floral differentiation between outcrossing and (largely or partially) selfing species, but we note that it is possible that the ability of (some) homostylous species to reproduce both autogamously and allogamously plays a role in explaining the high variability of floral traits in homostylous species, in comparison to their

obligately outcrossing, heterostylous relatives.

Although a general relationship between the evolution of polyploidy and the loss of heterostyly has been suggested for *Primula* (Richards 2003; Naiki 2012), comparision of ploidy levels (following Richards 2003) of sampled homostylous species with those of related, heterostylous species revealed that polyploidy unlikely is a major confounding factor in our analyses. For transitions involving homostylous species of known ploidy level, homostylous species usually were either exclusively diploids (*P. mollis, P. septemloba, P. simensis+P. verticillata*), or transitions to homostyly gave rise to a clade of diploid and polyploid species (*P. chungensis+P. cockburnia+P. japonica*) or to a polyploid (*P. watsonii*) that was sister to a clade containing both diploid and polyploid, heterostylous species. In one case, heterostylous, diploid species were sister to homostylous, polyploid species, but flower size of these homostylous species (*P. halleri, P. laurentiana, P. scandinavica,* and *P. scotica*) was either smaller or larger than that of related, heterostyous species (Fig. 1). Thus, our data show no strong relationship between the transition to homostyly and the evolution polyploidy, nor a trend of homostylous, polyploid species being larger or smaller than related diploid, heterostylous species.

While current analytical approaches do not allow us to discern whether the signal of strong macro-evolutionary drift, σ^2, in homostylous species (Table 2) reflects a high variability in selective optima, θ, among homostylous species or temporally fluctuating optima within and among homostylous lineages, they nevertheless enable us to conclude that homostylous species display considerable phenotypic variation - more than predicted by the paradigm of selfing as being typically associated with reduced floral size in self-compatible lineages. Additionally, differential strength of selection among tree partions (i.e. multiple α) is generally difficult to detect when tree partitions span unequal amounts of evolutionary time (Beaulieu et al. 2012), as in the current dataset (Fig. 1), and co-estimation of multiple α and σ^2 proved not possible (see methods). Therefore, the higher levels of stochasticity, σ^2, detected by the two best models for the evolution of corolla-tube length and corolla-limb diameter after the loss of heterostyly (Table 2) suggest that the evolutionary trajectory of these traits is likely to include values that are more extreme in homostyles, irrespective of whether the microevolutionary process is driven by increased genetic drift, decreased selection, or a combination of both.

Because higher selfing rates, as expected for homostyles compared to heterostyles, will generally lead to lower effective population sizes, genetic drift is indeed likely to become stronger after a transition to homostyly (Lloyd 1980; Hamrick and Godt 1996). Moreover, the increased auto-fertility of homostylous compared to heterostylous species implies that homostylous species rely less on pollinators for successful reproduction. Therefore, pollinators would exert less stabilizing selection on the floral traits of homostylous species compared to heterostylous species (Cresswell 1998), an expectation congruent with the empirical finding that levels of floral integration may decrease after self-incompatibility is lost (Anderson and Busch 2006). Thus, changes in floral traits involved in pollinator attraction (e.g., corolla-limb diameter) and interaction (e.g., corolla-tube length) are likely to become more easily fixed by neutral processes in homostylous than in heterostylous species, a prediction that could explain the relatively wide variation of such floral traits in homostylous species (Figs 1, 2, 4). At the same time, the lower effective population size of selfing species further implies

that the efficacy of selection should increase (Lloyd 1980; Hamrick and Godt 1996), meaning that achieving adaptation to a new fitness optimum may proceed faster in species without heterostyly (Glémin and Ronfort 2012). To summarize, both neutral and adaptive processes affecting the evolution of floral morphology are likely to proceed at a higher rate in homostylous compared to heterostylous species. Indeed, *Primula* species that have much longer floral tubes than other species of their section are usually homostylous (e.g. *Primula halleri* vs. other species of Section *Aleuritia*, or *P. verticillata* vs. other species of Section *Sphondylia*; Richards 2003). Moreover, multiple self-compatible lineages within the clade /Primula are morphologically so aberrant that they are frequently recognized as separate genera (i.e. *Dodecatheon, Cortusa, Sredinskaya*), whereas this is the case for only one group of self-incompatible species (i.e. *Dionysia*; Scott 1865, Richards 2003, Mast et al. 2006, Reveal 2009).

A combination of of genetic drift and relaxed selective constraints in homostylous species might also explain the counterintuitive results of posterior estimates of some traits (e.g., calyx length and corolla-tube length) being overall larger in homostylous than heterostylous species (Figs 3, 4), even though their selective optima (θ) are slightly smaller in homostylous species (Table 2), provided that the posterior mean in the MCMCglmm analysis is not more strongly affected by outliers than θ in evolutionary models (which would make it a currently unknown methodological artifact). If, upon the evolution of homostyly, the optimal trait value shifts to a (slightly) smaller value, congruent with a shifted resource-allocation optimum (Sicard and Lenhard 2011), but the stochastic fluctuations increase so much that some species evolve very large trait values (as for instance evidenced by the triangles in the bottom left quadrant of Fig. 2), then the net effect of homostyly, as measured by the MCMCglmm analysis (Fig. 3), would be shifted toward increased trait size.

To summarize, the release of evolutionary constraints on flowers after the loss of heterostyly (exemplified by higher levels of σ^2 for most traits in homostylous species), combined with the lack of strong selective pull towards new trait optima (exemplified by similar values for α in homostylous and heterostylous species or a single, global α; Table 2), are likely to profoundly affect the trajectory of floral evolution in homostylous primroses. This conclusion contrasts with the theoretical predictions of Glémin and Ronfort (2012), who argued that directional selection toward a new optimum of resource allocation within flowers would be required to explain evolutionary trajectories upon transitions toward high selfing. Extreme empirical cases of a strong selfing syndrome evolving over short evolutionary timespans, such as in the well-studied systems *Capsella rubella* and *Leavenworthia alabamica*, may indeed be triggered by strong positive selection (Foxe et al. 2009; Guo et al. 2009; Busch et al. 2011; Sicard et al. 2011; Slotte et al. 2012), but our results suggest that the evolutionary fate of homostylous primroses is much more variable due to either an increase in the strength of genetic and macro-evolutionary drift and/or a release of selective constraints from pollinators, or alternatively by the adoption of several, distinct evolutionary regimes among homostylous species. To put it simply: while some species evolve a typical selfing syndrome after the transition to homostyly (e.g., *P. cicutariifolia, P. prenantha, P. muscoides, P. watsonii*), others do not (e.g., *P. halleri, P. japonica, P. mollis*; Fig. 1).

Concluding, our findings imply that the reduction of floral size in partially or mostly selfing species is not a general law. Rather, it represents one of several possible outcomes of the loss of self-incompatibility. This view is congruent with Ernst's (1962, p.94) characterization of the transitions in

morphological and reproductive characters associated with the loss of heterostyly as "an overall picture of surprising diversity of floral plasticity" (translated from German by JdV), while summarizing his forty-five years of work on the breeding systems of *Primula*. Though data is currently lacking, it would be useful to determine whether pollen/ovule ratios display a similar variability in homostylous species, as lower pollen/ovule ratios are typically considered part of the selfing syndrome (Ornduff 1969; Cruden 1977; Ritland and Ritland 1989). It would also be interesting to investigate why the loss of self-compatibility is sometimes associated with the evolution of smaller flowers and sometimes with the evolution of larger flowers. Does this variation reflect contrasting outcomes of truly neutral genetic drift, or do selective regimes differ among species? Compelling evidence for either scenario will probably stem from a combination of new comparative methods with targeted experiments on reproductive ecology.

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Supplementary information, Table S1. Model fit and estimated parameters of all models of quantitative-trait evolution for the four floral traits, indicating the fit of the model to the data (corrected AIC score, AICc), probability that the model is the best model among the candidate models (AICc weight), and estimated values of the parameters θ (theta; optimum), α (alpha, selective pull) and σ^2 (sigma^2; rate of random drift). When models contain a single, global parameter, estimates are italicized and printed in the center of the column; estimates for heterostyly- and homostyly-specific parameters are reported in their respective columns. NA: parameter not present in model. Results based on stochastic maps for the evolution of heterostyly generated under ARD-model (first page) and SYM-model (second page).

Trait	Model	AICc	AICc weight	*Theta*		*Sigma^2*		*Alpha*	
				Heterostyly-specific	Homostyly-specific	Heterostyly-specific	Homostyly-specific	Heterostyly-specific	Homostyly-specific
Character mapping using ARD-model									
Calyx length	BM_1	44.266±0.000	0.000±0.000	*0.751±0.000*		*0.433±0.000*		NA	
	BM_S	45.511±0.146	0.000±0.000	*0.743±0.001*		0.023±0.000	0.025±0.001	NA	
	OU_1	12.642±0.000	0.197±0.007	*0.684±0.000*		*0.958±0.000*		*6.351±0.000*	
	OU_M	11.446±0.039	0.354±0.011	0.623±0.008	0.615±0.008	*0.046±0.000*		*0.309±0.000*	
	OU_{MA}	12.724±0.143	0.183±0.009	0.616±0.008	0.617±0.009	*0.046±0.000*		0.314±0.002	0.316±0.001
	OU_{MV}	12.070±0.266	0.267±0.021	0.620±0.008	0.613±0.008	0.049±0.001	0.055±0.002	0.306±0.001	
Corolla-limb diameter	BM_1	246.270±0.000	0.000±0.000	*1.255±0.000*		*2.153±0.000*		NA	
	BM_S	246.102±0.186	0.000±0.000	*1.246±0.001*		0.127±0.004	0.125±0.004	NA	
	OU_1	173.267±0.000	0.234±0.005	*1.278±0.000*		*60.242±0.000*		*136.543±0.000*	
	OU_M	173.256±0.003	0.235±0.005	1.281±0.001	1.281±0.001	*2.869±0.014*		*6.503±0.031*	
	OU_{MA}	175.753±0.254	0.110±0.010	1.281±0.004	1.284±0.004	*0.332±0.006*		0.631±0.014	0.632±0.014
	OU_{MV}	172.070±0.071	0.421±0.008	1.283±0.001	1.282±0.001	2.780±0.155	2.757±0.125	*4.802±0.192*	
Herkogamy	BM_1	26.371±0.000	0.000±0.000	*0.510±0.000*		*0.376±0.000*		NA	
	BM_S	20.299±0.467	0.000±0.000	*0.521±0.001*		0.030±0.002	0.029±0.002	NA	
	OU_1	-15.705±0.000	0.000±0.000	*0.481±0.000*		*0.891±0.000*		*7.726±0.000*	
	OU_M	-63.575±0.160	0.013±0.011	0.303±0.023	0.298±0.023	*0.140.0.006*		*2.049±0.087*	
	OU_{MA}	-69.210±1.553	0.035±0.009	0.280.0.025	0.319±0.026	*0.090.0.003*		1.847±0.071	1.851±0.071
	OU_{MV}	-83.408±0.427	0.953±0.014	0.313±0.024	0.290.0.024	0.238±0.030	0.186±0.024	*5.073±0.351*	
Corolla-tube length	BM_1	238.612±0.000	0.000±0.000	*1.662±0.000*		*2.026±0.000*		NA	
	BM_S	232.143±0.191	0.000±0.000	*1.613±0.004*		0.136±0.006	0.143±0.006	NA	
	OU_1	202.254±0.000	0.029±0.002	*1.517±0.000*		*4.249±0.000*		*6.228±0.000*	
	OU_M	201.435±0.057	0.048±0.004	1.411±0.015	1.406±0.015	*0.206±0.000*		*0.304±0.000*	
	OU_{MA}	202.261±1.186	0.256±0.016	1.431±0.017	1.416±0.016	*0.184±0.004*		0.233±0.010	0.234±0.010
	OU_{MV}	195.481±0.198	0.666±0.018	1.398±0.014	1.386±0.015	0.284±0.014	0.293±0.014	*0.282±0.001*	
Trait	Model	AICc	AICc weight	*Theta*		*Sigma^2*		*Alpha*	

Supplementary information, Table S1, continued.

Trait	Model	AICc	AICc weight	*Theta*		*Sigma²*		*Alpha*	
				Heterostyly-specific	Homostyly-specific	Heterostyly-specific	Homostyly-specific	Heterostyly-specific	Homostyly-specific
Character mapping using SYM-model									
Calyx length	BM_1	44.266±0.000	0.000±0.000	0.751±0.000		0.433±0.000		NA	
	BM_S	43.936±0.063	0.000±0.000	0.75.001		0.02.000	0.04.001	NA	
	OU_1	12.642±0.000	0.247±0.006	0.684±0.000		0.958±0.000		6.351±0.000	
	OU_M	11.578±0.056	0.424±0.005	0.686±0.003	0.525±0.005	0.047±0.000		0.313±0.000	
	OU_{MA}	14.138±0.478	0.157±0.003	0.692±0.002	0.521±0.005	0.045±0.000		0.301±0.005	0.305±0.005
	OU_{MV}	13.427±0.091	0.172±0.007	0.686±0.004	0.522±0.005	0.046±0.001	0.055±0.001	0.308±0.001	
Corolla-limb diameter	BM_1	246.27±0.000	0.000±0.000	1.255±0.000		2.153±0.000		NA	
	BM_S	242.205±0.323	0.000±0.000	1.252±0.001		0.097±0.004	0.351±0.010	NA	
	OU_1	173.267±0.000	0.269±0.005	1.278±0.000		60.242±0.000		136.543±0.000	
	OU_M	173.265±0.000	0.270±0.005	1.277±0.000	1.281±0.000	2.944±0.002		6.674±0.004	
	OU_{MA}	189.454±2.787	0.068±0.005	1.276±0.001	1.131±0.021	0.359±0.019		0.787±0.053	0.809±0.053
	OU_{MV}	172.511±0.080	0.393±0.008	1.278±0.000	1.274±0.002	2.041±0.073	3.692±0.126	4.964±0.164	
Herkogamy	BM_1	26.371±0.000	0.000±0.000	0.51.000		0.376±0.000		NA	
	BM_S	10.642±0.620	0.000±0.000	0.535±0.001		0.016±0.002	0.171±0.007	NA	
	OU_1	-15.705±0.000	0.000±0.000	0.481±0.000		0.891±0.000		7.726±0.000	
	OU_M	-63.019±0.145	0.000±0.000	0.515±0.008	0.068±0.008	0.163±0.007		2.38±0.106	
	OU_{MA}	-64.754±1.280	0.004±0.001	0.512±0.011	0.076±0.014	0.144±0.006		1.921±0.088	1.887±0.087
	OU_{MV}	-81.731±0.201	0.996±0.001	0.502±0.013	0.10.013	0.564±0.036	0.088±0.023	7.845±0.428	
Corolla-tube length	BM_1	238.612±0.000	0.000±0.000	1.662±0.000		2.026±0.000		NA	
	BM_S	222.382±0.191	0.000±0.000	1.643±0.002		0.082±0.004	0.395±0.007	NA	
	OU_1	202.254±0.000	0.005±0.001	1.517±0.000		4.249±0.000		6.228±0.000	
	OU_M	201.719±0.047	0.008±0.001	1.519±0.005	1.301±0.014	0.208±0.000		0.307±0.000	
	OU_{MA}	201.951±1.555	0.321±0.025	1.566±0.008	1.333±0.009	0.127±0.004		0.179±0.014	0.219±0.013
	OU_{MV}	191.999±0.162	0.666±0.025	1.52.006	1.28.009	0.163±0.008	0.604±0.010	0.268±0.000	

Results reported as mean ± standard error across 100 stochastic maps.

Chapter IV: Reproductive Implications of Herkogamy in Homostylous Primroses: Variation during Anthesis and Reproductive Assurance in Alpine Environments

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Reproductive implications of herkogamy in homostylous primroses: variation during anthesis and reproductive assurance in alpine environments

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Summary

1. Unreliable pollinator service is thought to promote the evolution of self-compatible plant breeding systems, because selfing may provide reproductive assurance when outcrossing opportunity is limited. The recurrent evolution of self-compatible homostyly from obligately outcrossing heterostylous species has been regarded as a classic example of evolutionary response to lack of pollinators or mates, as homostyly frequently occurs in pollinator-limited or marginal environments. However, male and female sexual organs of homostylous species may display spatial separation (herkogamy), an arrangement presumed to promote outcrossing. It is largely unknown to what extent variation in herkogamy affects opportunities for autonomous selfing and reproductive assurance in self-compatible, homostylous species.

2. Using the homostylous *Primula halleri*, restricted to alpine environments, we investigated whether herkogamy occurs and varies during anthesis, among individuals, and populations, and compared the effects of herkogamy on seed set among three experimental treatments, to elucidate how herkogamy affects reproductive strategies in a homostylous species.

3. Herkogamy decreases during anthesis, but the ultimate expression of herkogamy in mature flowers differs among individuals and populations. Caging caging experiments indicate that herkogamy reduces a plant's potential for autonomous selfing, and emasculation and open-pollination treatments demonstrate that herkogamy markedly decreases total seed set and the potential for reproductive assurance.

4. Herkogamy early in anthesis may enhance outcrossing potential, while its decrease later could enable reproductive assurance via delayed autonomous selfing in some, but not all plants. Conversely, pronounced herkogamy in older flowers comes at the cost of reduced total reproductive output and imposes pollinator dependence for reproduction, but may promote the genetic diversity of populations.

5. Our study suggests that even small amounts of herkogamy can have large effects on the reproductive strategy of homostylous species, by enabling more outcrossing than generally thought to be typical of homostyly.

Key-words: alpine/arctic, breeding system, delayed selfing, development, dichogamy, floral morphology, heterostyly, mixed mating, pollen limitation, *Primula*

Introduction

Scarcity of pollinator services has major consequences for plant reproduction and evolutionary processes, as recognized by early naturalists (e.g. Müller 1881; Schröter 1926). The pollinator fauna of alpine (i.e. above tree line) environments is generally depauperate, in terms of number of species and individuals, as compared to that of lower altitudes (e.g. Arroyo, Primack & Armesto 1982; Warren, Harper & Booth 1988). Moreover, the short flowering season and fluctuating weather conditions typical of alpine ecosystems further impair the reliability of pollinator services (Totland 1994; Bergman, Molau & Holmgren 1996; Körner 2003). Similar trends in pollination conditions occur with increasing latitude towards the poles (Hocking 1968; Kevan 1972).

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When lack of pollinators limits outcrossing opportunity and reproductive output (García-Camacho & Totland 2009), autonomous selfing (i.e. autogamy unaided by pollinators) may boost total seed production (i.e. reproductive assurance; Eckert, Samis & Dart 2006). Similarly, autonomous selfing may be beneficial when mates are scarce, as during colonization processes (Baker 1955), and in geographically or ecologically marginal habitats (Lloyd 1980). Reproductive assurance has often been invoked as an explanation for the evolution of selfing from primarily outcrossing ancestors (Darwin 1876; Fausto, Eckhart & Geber 2001; Kalisz, Vogler & Hanley 2004; Eckert, Samis & Dart 2006; Moeller 2006), widely recognized as one of the most frequent evolutionary transitions in flowering plants (Stebbins 1950; Grant 1981). Despite the ecological and evolutionary importance of reproductive assurance, experimental demonstrations remain scarce (reviewed by Eckert, Samis & Dart 2006).

Selfing is often associated with negative fitness effects because of reduced survival and fertility of the offspring (inbreeding depression; Charlesworth & Charlesworth 1987; Charlesworth & Willis 2009), as well as with long-term negative consequences on the genetic variability and viability of populations, potentially representing an evolutionary dead end (reviewed by Takebayashi & Morrell 2001). Therefore, autogamy may decrease fitness when ovules and pollen that could otherwise be outcrossed are self-fertilized (i.e. gamete discounting; Herlihy & Eckert 2002; Eckert & Herlihy 2004). Discounting costs can be incurred when autonomous selfing takes place prior to or competing with outcrossing (Lloyd 1992), or when pollinators mediate selfing concurrently with outcrossing by foraging within flowers (facilitated selfing) or between flowers of the same plant (geitonogamy; Vaughton & Ramsey 2010). However, if autonomous selfing occurs at the end of floral life after opportunities for outcrossing have been exhausted (i.e. delayed autonomous selfing; Lloyd 1992), it affords the benefits of autogamy, while avoiding discounting costs, a 'best-of-both-worlds' scenario that seems ideally adaptive in alpine/arctic habitats (Kalisz & Vogler 2003; Moeller 2006; Duan *et al*. 2010; Vaughton & Ramsey 2010). The relative timing and mode of selfing and outcrossing events may thus be important for overall reproductive fitness and long-term evolutionary survival (Lloyd 1992; Eckert, Samis & Dart 2006; Vaughton & Ramsey 2010).

Plants have evolved a wide range of floral traits that may influence the dynamics of sexual reproduction, providing the morphological and physiological basis of plant reproductive strategies. The study of the function and loss of complex floral polymorphisms has supplied key model systems for understanding the evolution of selfing (Darwin 1877; Barrett 2003, 2010). A prime example is the evolution of self-compatible homostylous species from obligately outcrossing heterostylous species (Ganders 1979; Barrett 1992; Barrett & Shore 2008; Cohen 2010). Heterostyly is thought to promote cross-pollination and reduce selfing and sexual interference, via the reciprocal placement of male and female organs in different floral morphs and an incompatibility system that prevents pollen germination within the same flower or floral morph (Darwin 1877; Charlesworth & Charlesworth 1979; Ganders 1979; Barrett 1992; Barrett, Jesson & Baker 2000; Barrett 2002; Barrett & Shore 2008; Cohen 2010). Therefore, heterostylous flowers depend on pollinators and mates for sexual reproduction. Homostylous species evolved multiple times independently from heterostylous ancestors in many of the *c*. 28 plant families with heterostyly (e.g. in *Amsinckia*, Boraginaceae, Schoen *et al*. 1997; *Houstonia*, Rubiaceae, Church 2003; *Narcissus*, Amaryllidaceae, Graham & Barrett 2004; Pontederiaceae, Kohn *et al*. 1996; *Primula*, Primulaceae, Mast, Kelso & Conti 2006; *Turnera*, Turneraceae, Truyens, Arbo & Shore 2005). Homostylous species have only one floral morph (i.e. monomorphic) and are self-compatible; hence self-fertilization is possible (reviewed by Ernst 1962; Ganders 1979; Barrett 1992; Barrett & Shore 2008; Cohen 2010). Although the term homostyly is sometimes applied to any plant species with stigmatic surface and pollen presentation at the same level, we refer here exclusively to monomorphic species that evolved within the context of heterostylous groups.

Because of the advantages of selfing, homostylous species have been hypothesized to be more successful than heterostylous relatives under ecological conditions that limit pollinator abundance, visitation activity or mate density (e.g. in *Amsinckia*, Ganders 1975; Plumbaginaceae, Baker 1966; *Primula*, Kelso 1992; Richards 2003; Guggisberg *et al*. 2006; *Psychotria*, Sakai & Wright 2008; *Turnera*, Barrett & Shore 1987). However, spatial separation between male and female sexual organs (i.e. herkogamy; Webb & Lloyd 1986) has been reported in several homostylous species (e.g. in *Primula*, Ernst 1962; Al Wadi & Richards 1993; Tremayne & Richards 1993; *Amsinckia*, Johnston & Schoen 1996; *Turnera*, Barrett & Shore 1987; *Narcissus*, Medrano, Herrera & Barrett 2005; Larrinaga *et al*. 2009). Herkogamy can negatively affect the relative selfing rate (e.g. shown in *Aquilegia*, Brunet & Eckert 1998; Herlihy & Eckert 2007; *Clarkia*, Holtsford & Ellstrand 1992; *Datura*, Motten & Stone 2000; *Mimulus*, Karron *et al*. 1997; *Nicotiana*, Breese 1959; *Turnera*, Belaoussoff & Shore 1995; but see Medrano, Herrera & Barrett 2005 on *Narcissus*), as it may decrease autonomous or facilitated selfing (Webb & Lloyd 1986; Barrett 2002). Importantly, herkogamy is usually heritable and may thus respond to selection (e.g. Shore & Barrett 1990; Lennartsson *et al*. 2000; Herlihy & Eckert 2007; Bodbyl Roels & Kelly 2011). However, it remains unclear exactly how variation in herkogamy may influence the potential for autonomous selfing under conditions of limited pollinator availability (Moeller 2006).

In primroses (*Primula*), the classic model for homostyly (Scott 1865; Darwin 1877), homostylous species have been predicted to be better adapted than their heterostylous relatives to the ecological settings typical of alpine and arctic environments (e.g. Kelso 1992; Richards 2003; Guggisberg

et al. 2006; Carlson, Gisler & Kelso 2008; Guggisberg, Mansion & Conti 2009). However, the potential role of herkogamy on the reproductive behaviour of homostylous primroses has never been considered: the alpine *Primula halleri* J.F.Gmel. (Fig. 1) provides an ideal study system to investigate it. *P. halleri* represents a classic example of the loss of heterostyly in alpine environments (e.g. Darwin 1877; Schröter 1926; Richards 2003), and extensive crossing experiments conclusively demonstrated that it is self-compatible (Ernst 1951). Early studies reported the occurrence of herkogamy in the species (Schröter 1926) and remarked that it may vary during floral anthesis (Ernst 1925). It is thus conceivable that this developmental variation, if sufficiently large to affect floral function, might offer contrasting mating opportunities at different stages of anthesis, including the possibility of delayed autonomous selfing. Mating opportunities that shift with the age of a flower have been mentioned in several species, although experimental evidence is generally limited (reviewed by Marshall *et al.* 2010).

The present study addresses the current gap of knowledge on how variation in herkogamy may affect pollinator dependence and opportunity for reproductive assurance in homostyly. The traditional focus on the proximity of sexual organs and self-compatibility has led to an interpretation of homostylous taxa as being primarily selfing and adapted to unreliable pollinator services, while the possible effects of herkogamy have been largely overlooked. Using *P. halleri* as our study system, we test whether: (i) the phenotypic expression of herkogamy changes during anthesis and variation between individuals and populations occurs and (ii) herkogamy affects seed set in open-pollinated, caged and emasculated plants. More broadly, the present study examines the effects of intraspecific and developmental variation in sexual organ distance on different components of plant reproductive success and contributes to understanding plant reproductive strategies in alpine environments.

Fig. 1. Inflorescence of *Primula halleri* showing developmental variation in herkogamy among flowers. Several flowers were removed, and the remaining flowers were opened longitudinally to expose the position of the sexual organs (stigma: ♀; anthers: ♂). The relative ages of the remaining flowers are indicated with letters 'a' (first flower that opened) to 'g' (youngest bud); flower 'e' represents an incompletely opened flower, in which anthesis is about to commence and anthers are about to dehisce. Scale bar indicates 1 cm. Note that the style extends beyond the anthers more pronouncedly in younger (centre of inflorescence) than in older, open flowers (periphery of the inflorescence), illustrating the general trend that herkogamy decreases with floral age.

Materials and methods

STUDY SPECIES

Primula halleri J.F.Gmel. (synonym *Primula longiflora* All.; sect. *Aleuritia* Duby; Richards 2003) is a herbaceous perennial bearing 3–19 flowers in an umbel (Fig. 1), with one or two flowers opening per day (J. M. de Vos and S. T. Isham, pers. obs.). The anthers, attached to a short filament, are positioned *c.* 1 mm below the mouth and dehisce when the corolla opens. The stigma is placed either among, above (protruding 1–4 mm, occasionally up to 8 mm) or, rarely, below the anthers (see below; Ernst 1925). Flowers have simultaneous male and female phases (no dichogamy; Lloyd & Webb 1986; Lennartsson *et al.* 2000; Isham 2010; see Fig. S1, Supporting Information). Flowers wilt after 6–12 days of anthesis; manual pollination does not induce wilting. Because flowers develop sequentially, a single scape can bear open flowers for up to 3 weeks. The hummingbird-hawkmoth (Sphingidae: *Macroglossum stellatarum* Linnaeus, 1758) is the main pollinator (Schulz 1890), but appears to visit flowers very infrequently (observed briefly on three of 25 consecutive days of field monitoring; J. M. de Vos and S. T. Isham, pers. obs.). *Primula halleri* occurs in the Alps, Carpathian mountains and Balkan region, between (1000-) 1800 and 2400 (-2900) m (Lüdi 1927); its closest relative is the heterostylous, largely self-incompatible *Primula farinosa* (Guggisberg *et al.* 2006; Guggisberg, Mansion & Conti 2009).

After initial surveys in 2008, three populations (named A, B and C; see Table 1) of *P. halleri* in the Swiss Alps (Central and Upper Canton Valais) were selected as study sites. All populations occurred on steep (20°–45°), south facing, nutrient-poor grassy slopes on limestone at 2300–2350 m altitude, where the species is most abundant (Lüdi 1927). Populations A and C included several thousand individuals at a density of up to *c.* 10 plants m^{-2}; population B included *c.* 1000 individuals at slightly lower density. Populations were sufficiently distant from busy trails and pastures to minimize the possibility of anthropogenic disturbance. The rarity and recent decline of the species (Wohlgemut, Boschi & Longatti 2006) and time-consuming ascents restricted the number of populations that could be studied. All fieldwork was performed in June–September 2009.

PHENOTYPIC VARIATION IN SEXUAL ORGAN POSITION AND HERKOGAMY

Developmental variation during anthesis

We tested whether herkogamy changes during floral development using a regression approach in which the positions of sexual organs were modelled as a function of the number of days flowers had been open. Because precise measurements were only possible in fixed flowers, we harvested inflorescences in 70% EtOH when displaying a wide range of floral ages. We could determine relative floral age (i.e. opening order) from the position of flowers within each inflorescence, because they develop from the base to the top of the inflorescence and are positioned approximately in Fibonacci spirals (Fig. 1). Hence, the flower lowest in the inflorescence is the oldest flower, the one positioned at an angle of *c.* 110° from it is

Table 1. Interfloral variation among mature flowers for anther position, stigma position and herkogamy within populations A, B, C: mean trait values (±standard deviation, SD), results of Kruskal–Wallis tests for differences among plants and partitioning of variance among populations and among plants nested within populations (see text and Fig. 4)

	Population A		Population B		Population C		Variance components	
	Mean ± SD (mm)	Significance of variation among plants	Mean ± SD (mm)	Significance of variation among plants	Mean ± SD (mm)	Significance of variation among plants	Among populations, %	Among plants, %
Anther position	27·0 ± 2·8	χ^2 = 70·1, d.f. = 24, $P < 0{\cdot}001$	26·6 ± 2·2	χ^2 = 32·3, d.f. = 12, $P < 0{\cdot}001$	26·1 ± 2·1	χ^2 = 37·7, d.f. = 14, $P < 0{\cdot}001$	<0·01	87·3
Stigma position	28·5 ± 2·7	χ^2 = 65·9, d.f. = 24, $P < 0{\cdot}001$	27·2 ± 2·1	χ^2 = 32·3, d.f. = 12, $P = 0{\cdot}004$	25·9 ± 2·0	χ^2 = 35·7, d.f. = 14, $P = 0{\cdot}001$	20·1	65·2
Herkogamy	1·5 ± 1·4	χ^2 = 60·0, d.f. = 24, $P < 0{\cdot}001$	0·6 ± 1·3	χ^2 = 32·3, d.f. = 12, $P = 0{\cdot}001$	−0·2 ± 1·7	χ^2 = 37·1, d.f. = 14, $P < 0{\cdot}001$	24·3	54·8
Sample size (# flowers/# plants)	75 fl./25 pl.		39 fl./13 pl.		45 fl./15 pl.			
Locality	46°22·94′N/8°13·72′E (2300 m)		46°20·96′N/8°09·16′E (2300 m)		46°02·62′N/7°49·86′E (2350 m)			

the second oldest, etc. We determined the absolute age of each flower (number of days since the corolla first opened), by combining the relative age with detailed field observations on the number of open and closed flowers in each inflorescence on alternate days. To determine the positions of sexual organs, the corolla tube and calyx of each flower were slit longitudinally, opened, and a high-resolution digital image was taken with a Canon PowerShot A610 camera. The distance from the base of the ovary to (i) the base of the stigma ('stigma position') and (ii) the top of the anther ('anther position') were then measured to an accuracy of 0·01 mm using ImageJ 1.43 (http://rsbweb.nih.gov/ij/). The degree of herkogamy of each flower was calculated as the difference between (i) and (ii). Because the mean anther length of *P. halleri* was 2·4 mm (data not shown), the herkogamy values of flowers with stigmas between the anthers varied between 0 and *c.* −2·4 mm. We analysed only inflorescences in which ages of flowers could be determined unambiguously: 199 flowers from 25 individuals (4–18 flowers per inflorescence) of population A in total.

The statistical software R (v.2.14.1; R Development Core Team 2011) was used for all analyses in this study. To test whether herkogamy changed during anthesis, we applied a linear mixed effects model (LMM) fitted with restricted maximum likelihood (REML), implemented in nlme (Pinheiro *et al.* 2012). We treated floral measurement (i.e. both anther position and stigma position) as response variable. As fixed effects, we employed floral age, organ type (anther or stigma) and their interaction to test whether the positions of anthers and stigma changed with floral age and at different rates, thus causing changes in herkogamy during floral development. We used plant identity and flower identity nested within plant identity as random effects to account for variation between individuals and for hierarchical data structure. Finally, we calculated Spearman rank correlations between herkogamy and floral age to assess the rate and significance of decrease in herkogamy within each inflorescence, which allowed us to establish whether developmental changes in herkogamy differed between individuals.

Variation within and between populations

We investigated whether variation in anther position, stigma position, and herkogamy was explained by non-developmental, interfloral components in 75, 39 and 45 flowers, respectively, from randomly selected inflorescences of populations A, B, C (see Table 1). To correct for developmental variation during anthesis, we analysed flowers from the same developmental stage. We included only the three oldest, non-wilting flowers of each inflorescence (hereafter called 'mature flowers'), representing the ultimate expression of herkogamy. Positions of floral organs and herkogamy were determined as described above.

First, we used Kruskal–Wallis tests to assess whether the anther and stigma positions and the herkogamy of mature flowers differed significantly between individuals within populations. Secondly, we tested whether the examined floral traits differed significantly between populations by building a LMM for each population pair and floral trait. LMMs included one of the three floral traits as response variable, population as fixed effect and plant identity as random effect. Thirdly, we assessed how variances for anther position, stigma position and herkogamy are partitioned within and between populations by extracting the variance components for each floral trait from linear models fitted with REML, implemented in lme4 (Bates, Maechler & Bolker 2011). The models included population and plant identity nested within population as two random effects. Finally, we used two linear regression models to analyse correlations between herkogamy and floral organ positions, with herkogamy as response variable, population as a categorical effect and either anther or stigma position as quantitative predictor. Similarly, we used a third linear regres-

sion model to analyse the correlation between anther and stigma position among populations. We tested for heterogeneity in correlations among populations by including population × sexual organ interactions.

EFFECTS OF HERKOGAMY ON SEED SET

Experimental design

We evaluated whether interindividual variation in herkogamy affects reproductive assurance and pollinator dependence in *P. halleri*, by analysing total seed set of plants that differed in the herkogamy of mature flowers under four experimental treatments: open pollination (control), caging (i.e. pollinator exclusion), emasculation and caging + emasculation. Caged plants can only set seed via autonomous selfing, whereas emasculated plants can only set seed through outcrossing. Open-pollinated plants can potentially do both and can also produce seed via pollinator-mediated selfing. Plants that are both caged and emasculated can potentially produce seed asexually (i.e. apomixis). The occurrence of reproductive assurance can thus be determined by testing whether seed set is higher in open-pollinated than emasculated plants for a particular herkogamy class. If homostyly is associated with predominant selfing, as often presumed, we expect to find no significant difference in seed set between caged and control treatments, and higher seed set in control than emasculation treatments. If, on the other hand, herkogamy affects the reproductive strategy of homostylous species by diminishing selfing, we expect to find, as herkogamy increases, lower seed set in caged plants and a smaller difference in seed set between emasculated and control plants.

Data collection

To avoid damage to or accidental hand pollination of the flowers during manual measurements of herkogamy, individual plants were assigned to one of four herkogamy classes defined upon visual inspection of mature flowers in the inflorescence (see also Medrano, Herrera & Barrett 2005). Class assignment was congruent between two independent observers and on different days. We used the following herkogamy classes: 0, 1, 2 and 3–4 mm. Because anthers are invariably attached *c.* 1 mm below the corolla mouth, we could use the position of the stigma relative to the corolla mouth to assign emasculated flowers to the corresponding herkogamy classes.

Cages consisted of a chicken-wire frame covered by a fabric used to protect crop plants from pest insects and were employed in populations A, B, C. Emasculations were performed in unopened flowers near anthesis, by making a small longitudinal slit in the corolla and removing the undehisced anthers with tweezers. Treatments involving emasculation were performed in population A. To capture seeds, we bagged wilted flowers before capsules opened. Ripe capsules were collected at the end of the season. The number of seeds and undeveloped ovules were counted from a digital image or directly under a dissecting microscope. Seed set was expressed as the proportion of ovules that developed into seeds, thus allowing to correct for the large variation in ovule number, ranging between 197 and 493 ovules per flower. Accounting for loss of replicates because of the damage to the plants or abnormal fruit development, seed set could be determined in a total of 361 flowers from 76 plants; over 60 000 seeds and ovules were counted (see Table 2 for details on sample sizes).

Statistical analyses

We employed generalized linear mixed effects models (GLMMs), using a binomial error distribution with logit link function, because the response variable 'seed set' is a proportion. Population and plant identity nested in population were used as random effects. GLMMs were fitted using Penalized Quasi Likelihood, implemented in the R package MASS (Venables & Ripley 2002), because more than five observations were available for each group (Bolker *et al.* 2009). Significance was established using the Wald *t*-test, because it is robust to overdispersion (Bolker *et al.* 2009).

Table 2. Effects of herkogamy in mature flowers and open-pollination (control), caged (pollinator exclusion) and emasculation treatments on seed set; results of generalized linear mixed effect models. Top: mean seed set (± standard deviation) and treatment effect size (± standard error) within individual herkogamy classes and all herkogamy classes. Bottom: herkogamy effects within treatments, indicating significance. Seed set is expressed as the proportion of ovules in a flower that developed into seed. See also Fig. 5. Sample sizes: open pollination, 125 flowers/30 plants (10 plants per population); caging, 142 flowers/30 plants (10 plants per population); emasculation, 40 flowers/7 plants (1 population); emasculation plus caging, 40 flowers/9 plants (1 population)

	Open pollination (control)	Caged (pollinator exclusion)			Emasculation		
			Treatment effect: Control vs. pollinator exclusion			Treatment effect: Control vs. emasculation	
Herkogamy class	Mean seed set (median)	Mean seed set (median)	Effect size	Significance	Mean seed set (median)	Effect size	Significance
0 mm	0·82 ± 0·31 (0·94)	0·59 ± 0·43 (0·885)	−0·97 ± 0·61	$t_{23} = -1{\cdot}59$, $P = 0{\cdot}12$	0·35 ± 0·30 (0·26)	−2·77 ± 0·96	$t_{23} = -2{\cdot}9$, $P = 0{\cdot}008$
1 mm	0·74 ± 0·29 (0·815)	0·25 ± 0·36 (0·025)	−2·58 ± 0·54	$t_{16} = -4{\cdot}80$, $P < 0{\cdot}001$	0·22 ± 0·37 (0·06)	−2·60 ± 0·87	$t_{16} = -2{\cdot}99$, $P = 0{\cdot}009$
2 mm	0·66 ± 0·24 (0·68)	0·07 ± 0·20 (0·00)	−3·87 ± 0·93	$t_{10} = -4{\cdot}16$, $P = 0{\cdot}002$		No data	
3–4 mm	0·61 ± 0·27 (0·67)	0·00 ± 0·00 (0·00)	−4·81 ± 2·94	$t_{32} = -2{\cdot}10$, $P = 0{\cdot}044$	0·37 ± 0·39 (0·26)	−0·73 ± 0·50	$t_{32} = -1{\cdot}47$, $P = 0{\cdot}15$
Overall	0·74 ± 0·29 (0·89)	0·33 ± 0·41 (0·025)	−1·05 ± 0·50	$t_{60} = -2{\cdot}10$, $P = 0{\cdot}040$	0·32 ± 0·34 (0·23)	−0·46 ± 0·76	$t_{60} = -3{\cdot}26$, $P = 0{\cdot}002$
Herkogamy effect	−0·41 ± 0·16 ($t_{94} = -2{\cdot}55$, $P = 0{\cdot}012$)	−1·53 ± 0·41 ($t_{60} = -3{\cdot}72$, $P < 0{\cdot}001$)			0·36 ± 0·36 ($t_{60} = 1{\cdot}00$, $P = 0{\cdot}31$)		

We generated a first GLMM for open-pollinated plants to test whether herkogamy had a significant effect on total seed set. We then built a full GLMM that allowed us to simultaneously assess the relative effects of herkogamy, caging and emasculation on seed set, with open pollination as the control. The model included herkogamy class, treatment and treatment × herkogamy interactions as fixed effects. We tested whether herkogamy reduced seed set in caged plants, by determining the significance of the caging × herkogamy interaction term. The full model also allowed us to test whether emasculated plants set less seed than open-pollinated plants (emasculation effect) and whether the effect of emasculation differed among herkogamy classes (emasculation × herkogamy interaction). Additionally, we used a series of four GLMMs to test whether seed set differed significantly between control and caged and between control and emasculated plants for each of the four herkogamy classes. Finally, to check whether seeds can be formed apomictically in *P. halleri,* we used summary statistics to establish whether seed set occurred in plants that were emasculated and caged.

Results

PHENOTYPIC VARIATION IN SEXUAL ORGAN POSITION AND HERKOGAMY

Developmental variation during anthesis

The positions of both anthers and stigmas increased significantly during floral development (floral age: $t_{173} = 14{\cdot}463$, $P < 0{\cdot}001$), but at different rates (organ type × floral age interaction: $t_{197} = -8{\cdot}981$, $P < 0{\cdot}001$), causing a general and significant decrease of herkogamy throughout anthesis (Fig. 2). While anthers started in a lower position than stigmas (intercepts ± SE of 22·68 ± 0·45 and 25·89 ± 0·49 mm, respectively), the former raised their position faster than the latter (slopes of 0·65 ± 0·04 and 0·41 ± 0·05 mm day^{-1}, respectively; Fig. 2).

Herkogamy decreased in 22 of 25 inflorescences, as indicated by negative Spearman rank correlations, and the decrease was significant in eight cases at $\alpha = 0{\cdot}05$ (Fig. 3). In plants 13–25, no flowers reached a degree of herkogamy below 1 mm, while in plants 2–12, the oldest flowers had the stigma positioned among or just above the anthers. Only plant 1 presented one flower with the stigma below the anthers (Fig. 3).

Variation within and between populations

Anther position, stigma position and herkogamy of mature flowers differed significantly between individuals in populations A, B, C (all $P < 0{\cdot}005$; Table 1). Between populations, however, anther position did not differ significantly, while stigma position and herkogamy differed significantly between some, but not all population pairs (Fig. 4; Table S1, Supporting Information). The variance of the three floral traits was partitioned mostly among plants within populations (87·2–54·8%) rather than among populations (24·3 –<1%; Table 1). Stigma and anther positions were overall positively and significantly correlated with each other (Fig. S2; Table S2, Supporting Information). Overall, herkogamy was negatively correlated with anther position and positively, but not significantly, with stigma position. The correlations did not (anther position and herkogamy) or did (stigma position) significantly differ among populations.

EFFECTS OF HERKOGAMY ON SEED SET

Seed set was absent in 49 of 54 flowers from nine plants that were emasculated and caged. The proportion of ovules that developed into seed (seed set ± SD) from the remaining five flowers of four different plants was 0·254 ± 0·317 (range 0·03–0·77). This low seed set is likely explained by emasculation error rather than apomixis, for seed set varied among flowers between inflorescences, while in the case of apomixis, it is expected to be similar across flowers within an inflorescence.

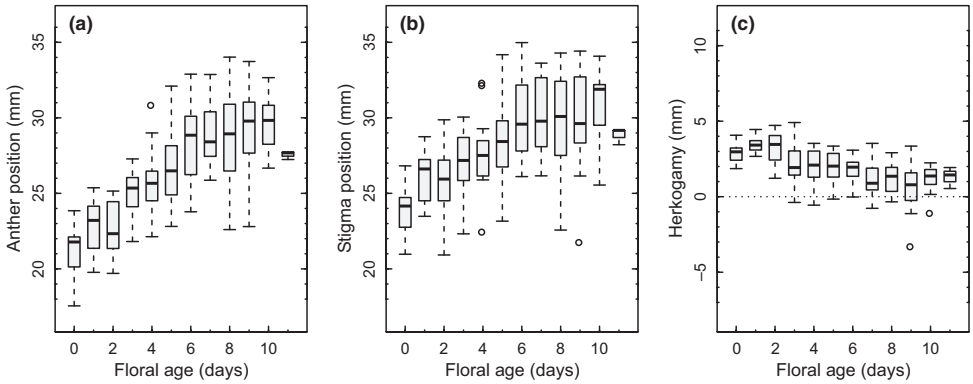

Fig. 2. Boxplots of variation in anther position (a), stigma position (b), and herkogamy (c) with floral age from 199 flowers of 25 plants in population A. The dotted horizontal line in panel (c) indicates a herkogamy level of zero mm (i.e. the point at which the stigma is positioned among the anthers). Anther position increases more rapidly than stigma position during development ($P < 0{\cdot}001$), contributing to the lower levels of herkogamy in older flowers.

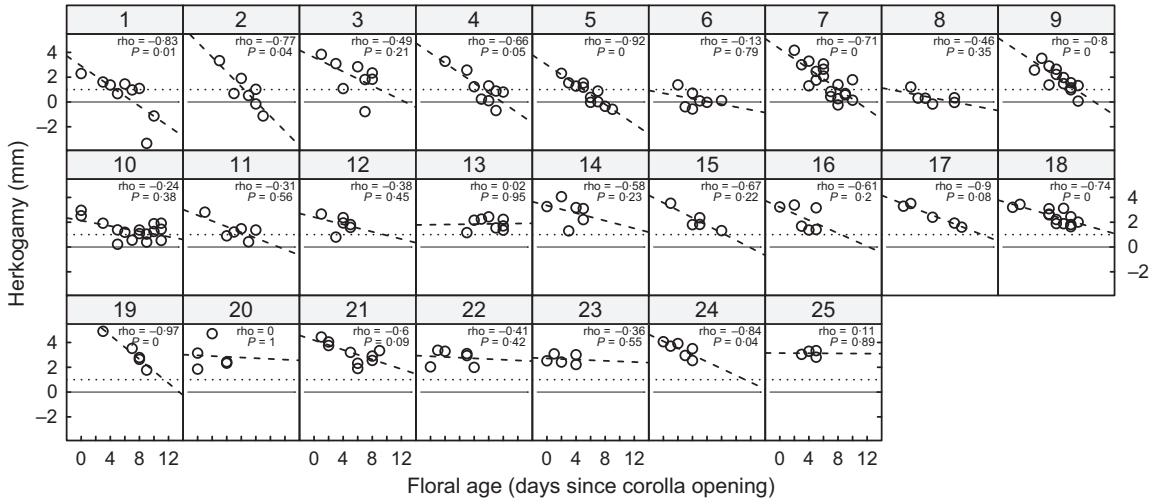

Fig. 3. Developmental variation in herkogamy throughout anthesis among 199 flowers of 25 plants from population A. Each scatter plot corresponds to one inflorescence and each circle to one flower. Dashed lines indicate the linear regression of herkogamy over floral age per inflorescence, with Spearman rho and *P*-value indicated. Horizontal solid lines indicate a herkogamy level of zero mm (i.e. the point at which the stigma is positioned among the anthers); dotted lines indicate the approximate maximal amount of herkogamy allowing autonomous selfing (see also Fig. 5). While herkogamy generally decreases with floral age, the minimum level of herkogamy reached during anthesis varies between individuals (see also Table 1).

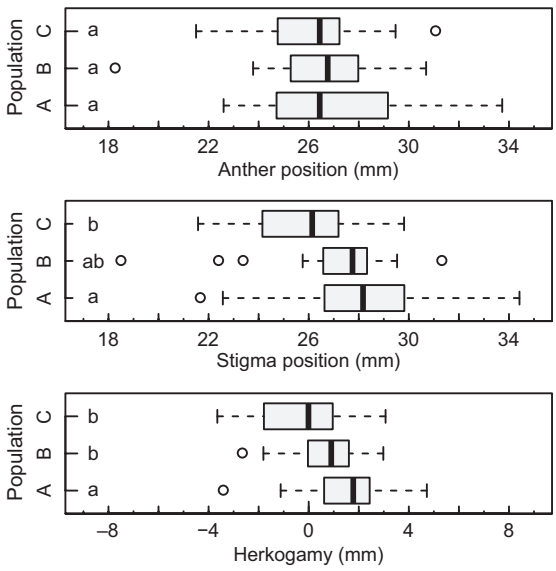

Fig. 4. Boxplots of variation in anther position, stigma position and herkogamy between populations A, B and C (159 mature flowers from 53 inflorescences; see Table 1); shared lowercase letters indicate no significant differences between populations at $\alpha = 0{\cdot}05$. Anther position does not significantly differ among populations, but stigma position and herkogamy do.

In open-pollinated plants, herkogamy correlated negatively with seed set ($P = 0{\cdot}012$; Table 2; Fig. 5). The full GLMM indicated that seed set in caged (selfing only) and emasculated plants (outcrossing only) was significantly lower ($P = 0{\cdot}04$ and $P = 0{\cdot}002$, respectively) than in open-pollinated plants (selfing plus outcrossing). The interaction terms indicated that seed set decreased significantly with increasing herkogamy in caged plants ($P < 0{\cdot}001$), but not in emasculated plants ($P = 0{\cdot}31$). The four GLMMs within herkogamy classes indicated that seed set did not significantly differ between control and caged treatments when the stigma was placed between the anthers (herkogamy class 0 mm, $P = 0{\cdot}12$; Table 2, Fig. 5). However, seed set was significantly higher in the control than in the caged treatment when herkogamy was >0 mm and the difference increased with herkogamy (effect size from −2·58 to −4·81), suggesting that the proportion of autonomous selfing that might contribute to total seed set in open-pollinated plants decreases with increasing herkogamy. Seed set was significantly higher in open-pollinated than in emasculated flowers of the two lowest herkogamy classes ($P = 0{\cdot}008$ and $P = 0{\cdot}009$, respectively), but it did not significantly differ between the two treatments in the highest herkogamy class ($P = 0{\cdot}15$), implying that, at lower levels of herkogamy, seed in open-pollinated plants is not exclusively produced via outcrossing (Table 2, Fig. 5).

Discussion

We here document a general significant trend of decreasing herkogamy during anthesis in the homostylous *Primula halleri* (Figs 1–3), a classic example of the loss of heterostyly in alpine habitats (Scott 1865; Darwin 1877; Schröter 1926; Mast, Kelso & Conti 2006). We also establish that herkogamy varies between individuals and between populations in this species (Figs 3 and 4). Variation in herkogamy among individuals (e.g. Karron *et al.*

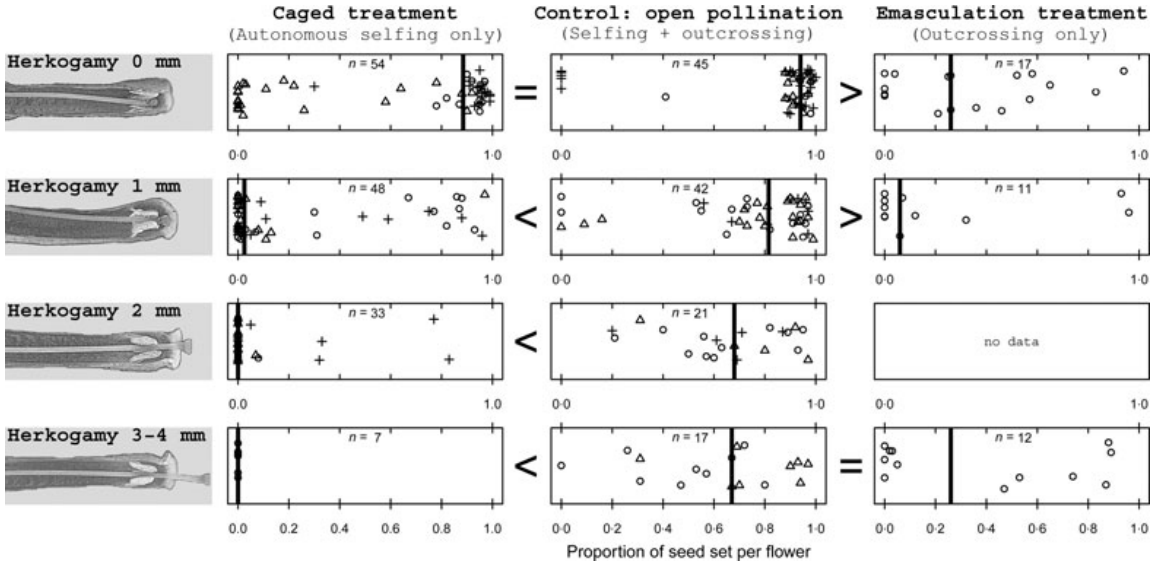

Fig. 5. Seed set across four herkogamy classes (rows) and three treatments (columns) in *Primula halleri*: caged treatment (pollinator exclusion, autonomous selfing only); control treatment (open-pollination, selfing and outcrossing); and emasculation treatment (outcrossing only). Circles, triangles and crosses represent seed set (expressed as the proportion of ovules per flower that developed into seeds) in populations A, B and C, respectively. Vertical bars indicate median seed set in each treatment/herkogamy panel; sample size (# flowers) is reported in each panel. The symbols '<', '>' and '=' between two adjacent panels indicate that seed set in the left panel is significantly lower, higher (at $\alpha = 0{\cdot}05$, see Table 2) or not significantly different than seed set in the right panel. In all four herkogamy classes, anthers were placed *c*. 1 mm below the corolla mouth; this allowed us to assign herkogamy classes to emasculated flowers based on the position of the stigma relative to the corolla mouth. No data were available for the emasculation treatment of herkogamy class 2 mm.

1997; Brunet & Eckert 1998), among populations (e.g. Holtsford & Ellstrand 1992) or, like in *P. halleri*, at both levels (e.g. Medrano, Herrera & Barrett 2005; Herlihy & Eckert 2007) is also known to occur in other species, including homostylous *Turnera ulmifolia* (Barrett & Shore 1987) and *Amsinckia spectabilis* (Johnston & Schoen 1996). A few previous studies reported variation in herkogamy in homostylous primroses (e.g. *Primula bellidifolia*, Tremayne & Richards 1993; *P. verticillata*, Al Wadi & Richards 1993; see also Ernst 1962), but reproductive implications were not considered. Here, we discuss the consequences of variation in herkogamy for reproduction of the alpine, homostylous *P. halleri*.

DECREASE IN HERKOGAMY DURING ANTHESIS

Although developmental variation in the separation of male and female organs is thought to be relatively common in angiosperms, experimental evidence is limited (reviewed by Fenster & Martén-Rodríguez 2007; Marshall *et al*. 2010). In *P. halleri,* the overall significant decrease in herkogamy was supported by the negative (but not always significant) regression slopes between sexual organ distance and floral age in 88% of the investigated inflorescences (Fig. 3). We were able to demonstrate that the faster increase in anther than stigma position during anthesis explains the lower herkogamy of older flowers (Figs 1 and 2). While some studies did not detect any changes of herkogamy during anthesis (e.g. Luijten *et al*. 1999; Medrano, Herrera & Barrett 2005; Larrinaga *et al*. 2009), other analyses, specifically on heterostylous and homostylous species, did, especially prior to anthesis (e.g. Stirling 1932; Li & Johnston 2010). Similarly to *P. halleri*, herkogamy significantly diminished during anthesis in *Gentianopsis paludosa* from the Qinghai–Tibetan Plateau (*c*. 3200 m; Duan *et al*. 2010), a pattern interpreted by the authors as preventing selfing and favouring outcrossing in younger flowers, while enabling self-fertilization in older ones (i.e. delayed selfing; Lloyd 1992). A comparable potential for delayed selfing was found among the large-flowered species of 20 investigated *Collinsia* and *Tonella* species (Armbruster *et al*. 2002).

In *P. halleri*, smaller separation between sexual organs at later stages of anthesis, coupled with lack of dichogamy (Isham 2010; Fig S1, Supporting Information), might also enable delayed autonomous selfing in older flowers. While herkogamy ranged between 2 and 4 mm in the first 2 days of anthesis (Fig. 2), enough to prevent seed set through autonomous selfing (caged treatment, Fig. 5; Table 2), about half of the analysed plants reached a level of herkogamy ≤ 1 mm at the end of anthesis (Fig. 3), the threshold at which autonomous selfing becomes possible (caged treatment; Fig. 5; Table 2). These plants thus might experience delayed selfing, proposed to be highly adaptive in alpine environments (Duan *et al*. 2010) where pollinator services are subject to high stochasticity (e.g. Arroyo,

Armesto & Primack 1985; Bergman, Molau & Holmgren 1996; Arroyo *et al.* 2006).

Conversely, herkogamy was higher than 1 mm at all stages of anthesis in the other half of the plants analysed for developmental variation of this trait (Fig. 3), implying that such individuals rely on pollinators for reproduction throughout the entire period of anthesis. This conclusion is contrary to common expectations, because homostyly is usually interpreted as alleviating dependence on pollinators and mates (e.g. Baker 1966; Kelso 1992; Richards 2003). Interindividual variation of herkogamy also occurred in populations B and C (Table 1, Fig. 4) although their mean herkogamy was lower than in population A (Fig. 4), with possible consequences for the extent of delayed selfing and outcrossing in different populations. Observations on living plants further suggest that other homostylous primroses may also exhibit developmental variation in herkogamy (e.g. *Primula eximia*, *P. incana, P. japonica, P. laurentiana, P. scotica* and *P. verticillata;* see also Chen 2009 on *P. cicutariifolia*). As these species belong to five sections in three subgenera of *Primula* (Richards 2003), wider applicability of our results is suggested, but experimental confirmation is needed.

EFFECTS OF HERKOGAMY ON REPRODUCTIVE ASSURANCE

Overall, *P. halleri* appears to benefit from the reproductive assurance enabled by the ability to self, as the higher seed set of open-pollinated vs. emasculated plants indicates (Table 2, Fig. 5). Similarly, in *Collinsia verna,* open-pollinated flowers set more seed than emasculated flowers, because autonomous selfing increased total reproductive output, thus providing reproductive assurance (Kalisz, Vogler & Hanley 2004). Conversely, significant effects of emasculation on reproductive output were not always found in other species, suggesting that, in these cases, the contribution of selfing to total reproduction was not considerable (e.g. Eckert & Schaefer 1998; see also Herlihy & Eckert 2002; Eckert, Samis & Dart 2006).

Despite the overall importance of reproductive assurance in *P. halleri*, interindividual variation of herkogamy in mature flowers strongly influenced the magnitude of this phenomenon (Table 2, Fig. 5). With increasing herkogamy, the difference in seed set between emasculated and open-pollinated plants became smaller, and it was only significant for plants with herkogamy ≤ 2 mm (Table 2; Fig. 5), suggesting that plants in the highest herkogamy class (3–4 mm) may not experience reproductive assurance. The negative influence of high herkogamy on the amount of reproductive assurance relates to the diminishing effect of herkogamy on autonomous selfing, as median seed set decreased from 0·885 to almost zero in caged plants with herkogamy *c*. 1 mm (Table 2; Fig. 5). The strong effect of herkogamy on autonomous selfing further suggests that even low amounts of sexual organ separation early in anthesis may promote outcrossing, by keeping ovules available for cross-fertilization, although this benefit of herkogamy may be obscured if facilitated selfing is frequent (Vaughton & Ramsey 2010). To summarize, comparisons of seed set among different treatments in four herkogamy classes indicate that, in the homostylous *P. halleri*, plants with greater distance between sexual organs in mature flowers are likely to be mainly outcrossed. This interpretation is compatible with the results of genetic studies, which typically found positive correlations between outcrossing rates and herkogamy both within (e.g. Barrett & Shore 1987; Karron *et al.* 1997; Brunet & Eckert 1998; Herlihy & Eckert 2007) and between populations (e.g. Shore & Barrett 1990; Holtsford & Ellstrand 1992; Belaoussoff & Shore 1995), although the correlation was not always significant (e.g. *Narcissus*; Medrano, Herrera & Barrett 2005).

The higher total reproductive output associated with the lower herkogamy of mature flowers (Fig. 5; Table 2) may suggest that selection should favour plants with low herkogamy (Figs 3 and 4; Table 1). How then can we explain the great variation of herkogamy detected in *P. halleri*? Several explanations may be proposed. First, reduced fitness of selfed progeny may offset the advantages of higher total seed set associated with increased selfing in plants with lower herkogamy (Herlihy & Eckert 2002). Therefore, even small amounts of herkogamy may alleviate the potentially negative effects of inbreeding and promote genetic diversity of populations, considered beneficial for the long-term adaptive potential of species (reviewed by Takebayashi & Morrell 2001). Nevertheless, common garden experiments indicated no evidence of high inbreeding depression in *P. halleri* at the seed-germination stage (Ernst 1951), although exhaustive studies over the entire life cycle are lacking.

Secondly, the effects of variation in herkogamy on the actual mating system may fluctuate between years (Eckert *et al.* 2009), and the selective pressures on herkogamy may differ over time as a consequence of changes in pollinator conditions (Kulbaba & Worley 2008). Thus, plants that performed poorly in a particular year may outperform others in subsequent years. Moreover, the timing of snow melt in different patches within a populations may create phenological variation in flowering on small spatial scales (pers. obs.), with possible effects on pollinator availability and optimal floral morphology of different plants (Forrest *et al.* 2011). Hence, selective pressures may not unidirectionally drive towards a decrease in herkogamy.

Thirdly, herkogamy is a compound trait dependent on the positions of both male and female organs, which themselves may convey independent fitness effects, irrespective of their contribution to herkogamy. For instance, the position of anthers alone might affect the dynamics of pollen deposition on a pollinator and thus the patterns of pollen export (Harder & Barrett 1993). The total reproductive fitness associated with a particular herkogamy class thus depends on the reproductive effects of male organ position, female organ position and herkogamy at each stage of

anthesis. Following the arguments explained by Johnston *et al*. (2009), analysis of individual fertility components (the capacities to sire seed through selfing, through pollen export, and through pollen import) may reveal that total reproductive fitness among herkogamy classes may deviate from patterns inferred from seed set alone, because the optimal floral design may differ for each fertility component. Here, it is interesting to note that developmental variation of herkogamy in *P. halleri* depends on differential rates of change in anther and stigma position (Figs 1 and 2), whereas only stigma position varies significantly between populations (Fig. 4), suggesting that complex selective pressures may act on different components of herkogamy at different hierarchical levels (Herlihy & Eckert 2007).

While *Primula* has served as the paradigmatic system for the study of homostylous species within a mainly heterostylous genus since Scott's (1865) pioneering work (see also Piper, Charlesworth & Charlesworth 1984; Barrett & Shore 2008; Cohen 2010), our study emphasizes for the first time the key role of variation in herkogamy in the reproductive ecology of a homostylous species. Our results suggest that a small distance between male and female organs early in anthesis can increase outcrossing opportunity, whereas 'excessive' herkogamy in older flowers comes at the cost of reducing total reproductive output and imposing pollinator dependence. This study provides new evidence that the reproductive strategies of homostylous species, which are self-compatible and derived from obligate outcrossing, heterostylous relatives, may be more complex than previously anticipated.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Evidence for a lack of dichogamy in *Primula halleri*, based on Isham (2010).
Fig. S2. Correlations between herkogamy and anther position, herkogamy and stigma position, and between stigma and anther position.
Table S1. Results of linear mixed effect models of variation in anther position, stigma position and herkogamy between populations.
Table S2. Results of linear regression models of overall correlations of herkogamy with anther position and stigma position and between stigma and anther position and heterogeneity in correlations among populations.

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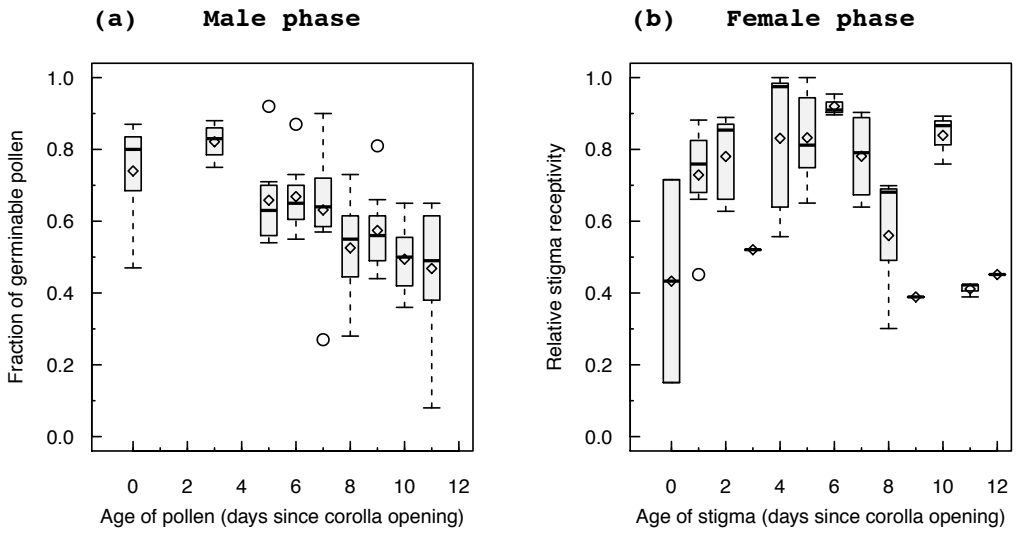

Figure S1. Boxplots of variation in male (a) and female (b) phases during anthesis in *Primula halleri*, based on Isham (2010)[‡], indicating that male and female phases fully: there is no evidence of dichogamy. (a) Anthers were collected from all flowers of one plant from population A when the range fully spanned anthesis. Pollen from each flower was mixed in seven separate drops of 10% sucrose solution on a petri dish, incubated for 24h at room temperature, placed in a freezer for 5 min, and mounted on a slide. Pollen germinability (male phase) was determined as the fraction of pollen grains that germinated in a sample of at least 200 pollen grains, when possible. In total, 68 observations from 10 flowers were available for analysis. To test if pollen loses its germination ability during anthesis, we used a GLMM with the ratio of germinated vs. non-germinated pollen grains as the response variable and a binomial model with logit link function. We included the age of the pollen in days since corolla opening (i.e., since anther dehiscence) as a fixed effect and flower identity as a random effect. Pollen grains retained their ability to germinate throughout anthesis, despite a slight but significant (t_8=- 5.61, $p<0.001$) decrease in germinability from 0.740±0.147 (mean of germinated fraction ± sd) at the day of anther dehiscence to 0.468±0.166 in the oldest, non-wilting flower. (b) Seven plants of population A were caged plus emasculated, and the ages of all flowers determined. Hand pollinations with a pollen mixture from freshly dehisced anthers of several donor plants were performed on all flowers of a plant when either the youngest flower opened or the oldest flower started wilting. Several hours later, flowers were harvested in FAA and transported to the lab, where stigmatic surfaces were stained for fluorescence microscopy. The number of germinated pollen grains per stigma was counted on 46 stigmas from seven plants. To test if a stigma lost its capacity to induce pollen germination as it aged, we used a GLMM with the number of germinated pollen grains as the response variable and a Poisson model with logarithmic link function. We included the age of the stigma in days since corolla opening as a fixed effect and plant identity as a random effect. Stigma receptivity (female phase) is plotted as the log of the number of germinated pollen grains on a stigma divided by the log of the highest observed value, hence, expressing it as a value between 0 and 1.The ability of a stigma to induce pollen germination did not significantly change throughout anthesis (t_{38}=-1.374, p=0.178). Diamonds indicate means.

[‡] Isham, S.T. (2010) Stigma receptivity, pollen viability, and outcrossing potential in the herkogamous homostylous species *Primula halleri*. Senior thesis, Department of Biology, The Colorado College, Colorado Springs.

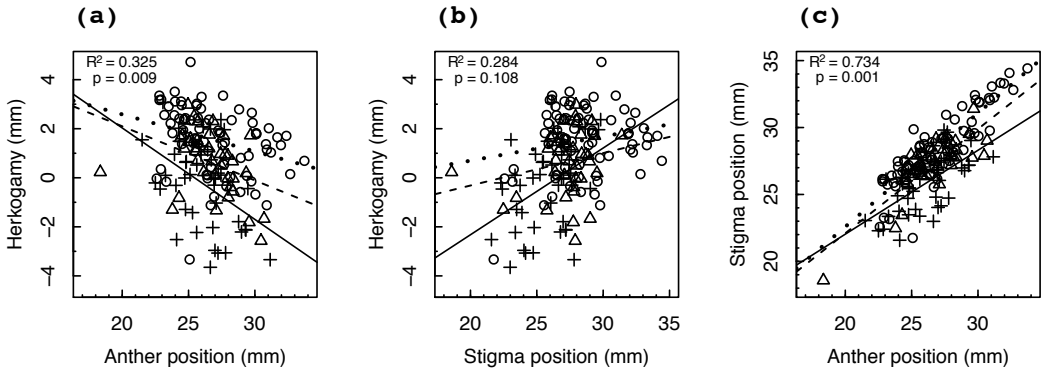

Figure S2. Correlations of herkogamy with anther position (a) and stigma position (b) and between stigma and anther position (c) from 159 flowers of 53 inflorescences in three populations. Circles, triangles and crosses represent the flowers of populations A, B, and C, respectively. Dotted, dashed, and solid lines represent linear regressions for the three populations, respectively. Multiple R^2 and p-values of overall correlations are indicated (see also Supporting Information Table S2).

Table S1. Variation in anther position, stigma position and herkogamy between populations A, B, and C, from 159 flowers of 53 inflorescences. Results of linear mixed effect models, indicating t-value (subscript: degrees of freedom) and p-value. See also Fig 4.

Population	Anther position		Stigma position		Herkogamy	
	A	B	A	B	A	B
B	t_{36}=-0.405, p=0.688		t_{36}=-1.52, p=0.138		t_{36}=-2.134, p=0.040	
C	t_{38}=-1.038, p=0.306	t_{26}=-0.631, p=0.534	t_{38}=-3.411, p=0.002	t_{26}=-1.867, p=0.073	t_{38}=-3.968, p<0.001	t_{26}=-1.608, p=0.120

Table S2. Overall correlations of herkogamy with anther position and stigma position and between stigma and anther position from 159 flowers of 53 inflorescences in three populations and heterogeneity in correlations among populations. Results from linear regression models, indicating coefficient ± standard error, t-value, degrees of freedom (df) and p-value. See also Supporting Information Fig. S2.

	Overall correlation				Heterogeneity of correlations among populations							
					Population A vs. B				Population A vs. C			
	coefficient	t	df	p	coefficient	t	df	p	coefficient	t	df	p
Anther position vs. Herkogamy	-0.151±0.057	-2.629	153	0.009	-0.070±0.115	-0.606	153	0.545	-0.222±0.116	-1.914	153	0.058
Stigma position vs. Herkogamy	0.099±0.061	1.618	153	0.108	0.034±0.124	0.271	153	0.917	0.254±0.122	2.093	153	0.038
Anther position vs. Stigma position	0.849±0.057	14.786	153	<0.001	-0.070±0.115	-0.606	153	0.545	-0.222±0.116	-1.914	153	0.058

CHAPTER V: PHYLOGENETIC ANALYSIS OF *PRIMULA* SECTION *PRIMULA* REVEALS RAMPANT NON-MONOPHYLY AMONG MOPRHOLOGICALLY DISTINCT SPECIES

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Phylogenetic analysis of *Primula* section *Primula* reveals rampant non-monophyly among morphologically distinct species

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Primula

ABSTRACT

The type section of *Primula* (Primulaceae), here considered to include seven species, is phylogenetically quite isolated in its genus. Although its species are popular ornamentals, traditional medicinal plants and model organisms for the study of heterostyly, the section has not yet been studied from a phylogenetic or evolutionary perspective. Using phylogenetic analysis of nuclear ITS and plastid data from all species and subspecies, we find that widespread *Primula elatior* is genetically heterogeneous and non-monophyletic to most if not all of the other ingroup taxa. The Genealogical Sorting Index (GSI) indicates that the assumption of all currently accepted species being independent lineages is consistent with the data. It is possible that *P. elatior* in its current circumscription may represents the disjointed remnant of an ancestral species from which the other recognized species diverged. However, based on available data, the alternative possibility of introgression explaining the non-monophyly of this species cannot be excluded. Species trees show *P. elatior* and *P. veris* as sister species. *Primula vulgaris* and *P. juliae* are closely related, while, in contrast to previous assumptions, *P. renifolia* does not appear to be a close relative of *P. megaseifolia*. With the section's isolation from the rest of the genus and very short internal branches, our dataset also presents a case study of the confounding effects of different branch length priors on the Bayesian estimation of resulting branch length estimates. Experimental runs using different priors confirm the problem of resulting estimates varying by orders of magnitude, while topology and relative branch lengths seem unaffected.

1. Introduction

Disentangling the evolution and phylogenetic relationships of closely related, hybridizing species is one of the most challenging tasks in systematics. Species in early stages of differentiation may lack or possess only to a limited degree some of the criteria for the identification of independent evolutionary lineages that are most commonly used, such as clear distinguishing characters, reproductive isolation, or reciprocal monophyly of gene trees. Lack of reciprocal monophyly can be caused by either ancestral polymorphism, when species are reproductively isolated but too young for lineage sorting to be complete, or introgression, if reproductive isolation is incomplete (Wendel and Doyle, 1998). The relative contributions of these two processes to lack of reciprocal monophyly remain very difficult to establish, despite recent advances provided by coalescent theory and population genetic approaches (Joly et al., 2009; Mims et al., 2010; Pelser et al., 2010).

Regardless of the underlying reason, non-monophyletic species complicate phylogenetic inference (e.g., Syring et al., 2007), as does incongruence between gene trees for different loci (e.g., Chen et al., 2009). Various approaches are now available for the reconstruction of species trees from gene trees, including, for example, minimizing deep coalescence (MDC; Maddison, 1997), Bayesian estimation of species trees (Liu and Pearl, 2007; Edwards et al., 2007; Heled and Drummond, 2010), and species tree estimation using maximum likelihood (Kubatko et al., 2009). However, all currently available methods assume that incongruence of gene trees is not explained by hybridization and demand data from a large number of independent loci, a requirement that is still difficult to meet for most non-model organisms (Hughes et al., 2006). The taxonomic treatment of non-monophyletic species is controversial, with opinions ranging from their outright rejection (Mishler, 1999; Ereshefsky, 2007) to the argument that the monophyly criterion is not applicable (Rieppel, 2010), or that 'wrong' taxonomy could explain non-monophyly (McKay and Zink, 2010). Conversely, under a view of species as distinctly evolving meta-population lineages (de Queiroz, 1999), different taxonomic circumscriptions can be compared statistically based on the likelihood of species trees constructed with coalescent-based methods (Carstens and Dewey, 2010; Yang and Rannala, 2010), and measures such as the Genealogical Sorting Index (Cummings et al., 2008) can be used to quantify the degree to which a lineage has achieved exclusive ancestry. In the present

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study, we discuss the case of a small, phylogenetically distinct clade of species that are poorly differentiated from a molecular point of view and explore the problems arising from the combination of phylogenetic isolation and lack of genetic differentiation among taxonomically distinct species.

Primula L. (Primulaceae) is a genus of perennial rosette plants with actinomorphic, sympetalous flowers. Including the phylogenetically nested *Dionysia* Fenzl., *Dodecatheon* L. and *Cortusa* L. (Mast et al., 2001; Trift et al., 2002; Martins et al., 2003), the group contains ca. 500 species and has a predominantly northern hemispheric distribution, with some representatives in Ethiopia and Southeast Asia and one isolated species in South America (Richards, 2003). Its center of diversity is the Central Asian mountain ranges, especially the Sino-Himalaya. Around 90% of the species are characterized by heterostyly (Richards, 2003), a condition in which populations consist of two floral morphs: "pins", with anthers in the lower and stigmas in the upper portion of the corolla tube, respectively, and "thrums" with a reverse arrangement of the sexual organs. This morphological differentiation is usually coupled with an incompatibility mechanism that hampers fertilization within the same morph (Barrett, 2002). Heterostyly is a genetically controlled breeding system that likely evolved to promote outcrossing (Barrett and Shore, 2008).

According to the most recent, global monographic treatment of *Primula*, the type section of the genus, sect. *Primula*, comprises six species that share some similarities with *P. grandis*, ascribed to its own section *Sredinskya* Stein (Richards, 2003; Fig. 1). Three species are widespread and well-known: the "primrose" *Primula vulgaris* Huds., comprising four mostly allopatric subspecies distributed through the Atlantic and Mediterranean parts of Europe, part of northern Africa and the Middle East; the "cowslip" *P. veris* L., with four subspecies and a Eurasian temperate distribution; and the "oxlip" *P. elatior* (L.) Hill., with eight subspecies and a very similar Eurasian distribution. Some of the current subspecies have previously been treated as segregate species (e.g., Komarov, 1963), especially colorful *P. elatior* ssp. *meyeri* (Rupr.) Valentine and J. Lamond, which was split into as many as three separate species, and *P. veris* ssp. *macrocalyx* (Bunge) Ludi, which was also sometimes recognized as a species (but see Länger and Saukel, 1993, for arguments in favor of reducing the number of subspecies in the section). The remaining three species have very restricted areas of distribution in the Caucasus and its immediate surroundings. The stoloniferous *P. juliae* Kusnez., together with *P. vulgaris* the presumed parent of a swarm of garden hybrids, occurs along the eastern part of the Caucasus chain, mostly in Georgia and Azerbaijan. *Primula megaseifolia* Boiss., an impressive plant with large leaves and a great number of purple flowers, is restricted to a thin coastal strip at the border of NE Turkey and Georgia. *Primula renifolia* Volgunov, restricted to the Dombai mountains of Cherkessk, directly north of the Russian–Georgian border, has the smallest distributional range in the section. Finally, *P. grandis* Trautv. occurs in the western part of the Caucasus chain. This species produces tall umbels of many pendent, tubular yellow flowers with strongly reduced corolla lobes and long-exerted stigmas (Fig. 1C). While sharing the same diploid chromosome number and pollen type with section *Primula*, *P. grandis* is morphologically so aberrant from the rest of *Primula* that it is traditionally treated as a separate monotypic section *Sredinskya*. However, recent molecular phylogenetic analyses firmly placed this species within sect.

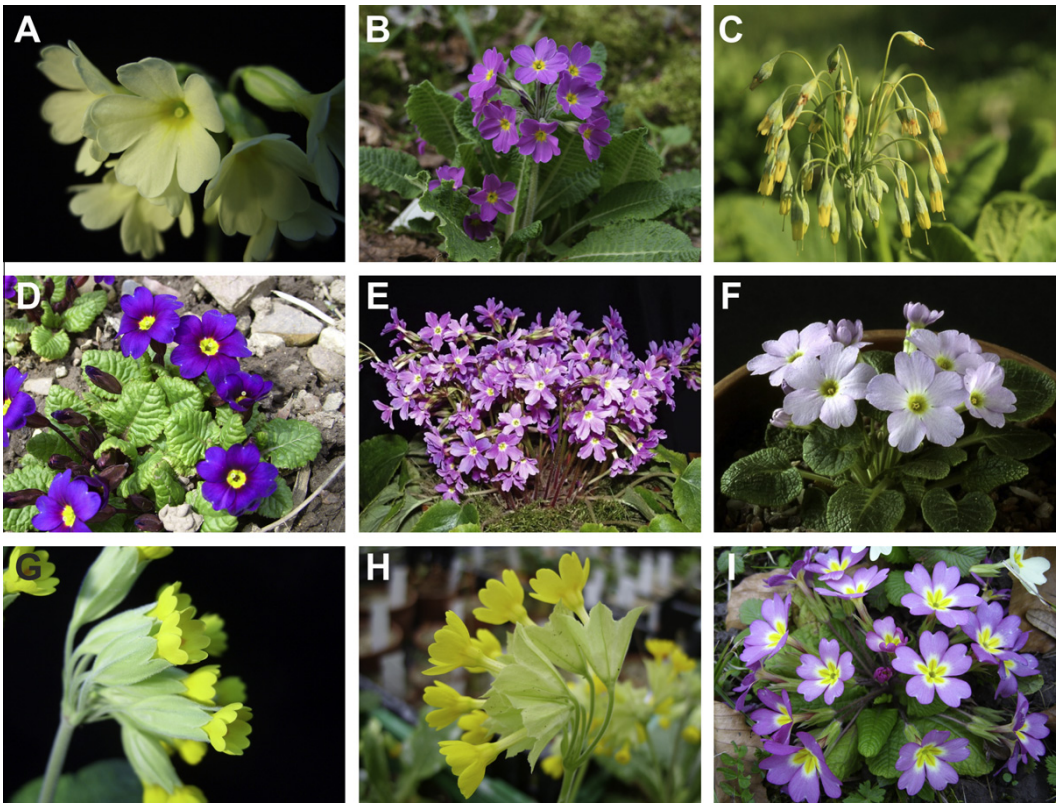

Fig. 1. The seven species of section *Primula*. (A) *P. elatior* ssp. *elatior*, (B) *P. elatior* ssp. *meyeri*, (C) *P. grandis*, (D) *P. juliae*, (E) *P. megaseifolia*, (F) *P. renifolia*, (G) *P. veris* ssp. *veris*, (H) particularly striking representative of *P. veris* ssp. *macrocalyx*, (I) *P. vulgaris* garden variety. Photo B courtesy of John and Wendy Mattingley, C published by Adrien Benoit à la Guillaume under the GNU Free Documentation License, E and F courtesy of Terry Mitchell, the others taken by the first author at the Botanical Garden of Zurich.

Primula, prompting us to consider it a seventh member of this section throughout the present study. All seven species are considered to be diploid, with a common chromosome number of $2n = 22$ (Richards, 2003), although values of $2n = 16–18$ have also been reported (Hayirlioglu-Ayaz and Inceer, 2003).

Hybridization is common in section *Primula* (Heslop Harrison, 1931; Smith et al., 1984; Richards, 2003; Gurney et al., 2007). Post-mating isolation mechanisms between various species pairs of section *Primula* have been studied experimentally in great detail (de Vries, 1919; Valentine, 1947, 1952, 1955). In the wild, hybridization between *P. vulgaris* and *P. veris* as well as *P. vulgaris* and *P. elatior* is prevalent when species co-occur (Woodell, 1965; Richards, 2003; Kálmán et al., 2004; B. Keller, personal observation). Despite the high frequency of hybrids, the extent of backcrossing and introgression into the respective species is unknown.

From a molecular phylogenetic point of view, *Primula* has recently been studied at the: (i) genus- or family-level in analyses of taxonomically broad datasets aimed either at improving the circumscription of the genus and its sections (Mast et al., 2001; Martins et al., 2003; Kovtonyuk and Goncharov, 2009) or elucidating the evolution of heterostyly (Mast et al., 2006); and (ii) intra-sectional level analyses of taxonomically narrow but in-depth datasets aimed at understanding evolutionary and biogeographic processes at the species level (Trift et al., 2004, on nested *Dionysia*; Mast et al., 2004, on nested *Dodecatheon*; Zhang et al., 2004, on sect. *Auricula*; Guggisberg et al., 2006, 2009, on sections *Aleuritia* Duby and *Armerina* Lindley; Kelso et al., 2009, on section *Parryi* W.W. Smith ex Wendelbo).

In family-level analyses of chloroplast and nuclear DNA sequences with low intra-sectional sampling, *Primula* section *Primula* (including *P. grandis*) formed a strongly supported clade subtended by a long branch, suggesting phylogenetic isolation from the section's closest living relatives, while the sampled species were subtended by extremely short internal branches, suggesting lack of inter-specific differentiation (Mast et al., 2001, 2006; Kovtonyuk and Goncharov, 2009). Despite the importance of this group as popular ornamentals, traditional medicinal plants, source of primrose wine, and for providing the model species for the study of heterostyly, hybridization and incompatibility (e.g., Darwin, 1877; Valentine, 1947, 1952), inter-specific relationships within the section and its evolutionary history remain unknown.

Conflicting views on the merit of assigning specific rank to various taxa in the section, the morphological plasticity of some currently accepted species and our ignorance of the frequency of introgression between them, as outlined above, prompted the present study. It thus had two related goals: (1) to examine whether the species of section *Primula*, and in particular the three widely distributed and morphologically heterogeneous species *P. elatior*, *P. veris* and *P. vulgaris* form independently evolving lineages, and (2) to provide a molecular phylogeny of the section. We performed extensive infra-specific sampling to investigate the degree to which species are genealogically diverged and analyzed both nuclear and plastid DNA sequences to examine whether the resulting phylogenies were congruent. A species phylogeny was produced with two different approaches. The degree to which species as currently circumscribed have achieved exclusive ancestry was assessed using the Genealogical Sorting Index (Cummings et al., 2008). Finally, we also considered the implications of our results for the taxonomic treatment of the study species.

2. Materials and methods

2.1. Sampling and extractions

Samples for molecular analysis were obtained from various sources, including garden collections, cultivars raised specifically for the present study from seeds sent by other botanical gardens, older collections of leaf tissue material either deep-frozen directly and stored at −80 °C or dried and stored on silica, and herbarium specimens of up to ca. 30 years of age. Before extraction, frozen or fresh samples were also first dried on silica. Sampling was designed to include all currently accepted species and subspecies of section *Primula*, and to cover as large a part of the distribution of the three widely distributed species as possible. In total, 65 ingroup samples were selected: 23 of *Primula elatior*, two of *P. grandis*, three of *P. juliae*, three of *P. megaseifolia*, one of *P. renifolia*, 19 of *P. veris*, and 14 of *P. vulgaris*. Eight outgroup species were sampled to represent the diversity of the large sister clade of section *Primula*, which includes several other sections and the genus *Dionysia* (Mast et al., 2006). For a detailed list of samples, see Appendix. In all cases, leaf fragments of up to 1 cm^2 were ground to dust using glass beads in a Retsch MM301 (Schieritz & Hauenstein, Arlesheim, Switzerland). Complete genomic DNA was then extracted using the DNeasy Plant Mini Kit (Qiagen AG, Hombrechtikon, Switzerland) following the manufacturer's instructions with minor modifications.

2.2. PCR and sequencing

Three DNA regions were sequenced for this study, one from the nuclear and two from the plastid genome. The nuclear ribosomal Internal Transcribed Spacer (ITS) region was amplified as described in Schmidt-Lebuhn (2008), but using a reaction volume of 25 μL and with the $MgCl_2$ concentration reduced to 70%. PCR products were purified with Exo I-CIAP (Fermentas GmbH, Le Mont-sur-Lausanne, Switzerland). PCR products of fifteen samples were purified with the NucleoSpin Extract II kit (Macherey-Nagel AG, Oensingen, Switzerland) and cloned using the CloneJET PCR Cloning Kit (Fermentas GmbH, Le Mont-sur-Lausanne, Switzerland) and self-made competent cells of strain DH5α to test for the existence of divergent paralogues. In all cases, eight clones were picked and sequenced after another PCR, following the same protocol as for direct PCR but using vector-specific primers. The plastid spacer regions *rps16-5′trnK*$_{UUU}$ (*rps16-trnK*) and *trnG*$_{UCC}$–*trnS*$_{GCU}$ (trnG–trnS) were amplified using the primers published by Shaw et al. (2007) and Hamilton (1999), respectively. The 25 μL reaction mix contained 1× PCR buffer, 33.75 nmol $MgCl_2$, 5 nmol dNTPs each, 2.5 pmol of both forward and reverse primer, 1 μL DMSO, and 5 μ Taq (Bioline, Luckenwalde, Germany). Cycler programs were run as described by Shaw et al. (2007). Sequencing reactions were prepared with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using 0.3 μL of the same primers as in the PCR amplification (10 μM), 1.0 μL BigDye terminator (version 3.1), 1.0 μL buffer and a total of 7.7 μL DNA-template and ddH_2O. Sequencing products were purified with Sephadex G-50 fine grade (GE Healthcare, Glattbrugg/Zürich, Switzerland) on 96-well multiscreen filtration plates (Millipore, Zug, Switzerland). Sequencing was carried out on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.3. Editing and sequence alignment

Sequences were edited using the BioEdit software (Hall, 1999) and Chromas Lite 2.01 (Technelysium pty. Ltd., Australia) and aligned using MUSCLE (Edgar, 2004) under default settings, with subsequent manual corrections. Alignments are available from the communicating author upon request. All three datasets were tested for the presence of recombination using the Phi statistic (Bruen et al., 2006) as implemented in the PhiPack software (Bruen, 2005) with default parameters but 10,000 permutations.

2.4. Phylogenetic analyses

2.4.1. Phylogenetic datasets

The phylogenetic relationships were predominantly estimated in a Bayesian framework, using MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). In total six datasets were analyzed, to explicitly address several potentially complicating factors. First, hybridization is suspected to play a role in the phylogenetic history of this group of species, as discussed in the introduction. This can potentially lead to incongruent topologies of plastid and nuclear gene trees (e.g. Van der Niet and Linder, 2008; Pelser et al., 2010). Therefore, analyses were conducted for chloroplast and nuclear markers both combined and separately. Secondly, as the branch to the ingroup (Section *Primula* including *P. grandis*) is comparatively very long (Mast et al., 2006), with the ingroup being sister to a large clade of over 200 species, we also analyzed the three marker combinations while excluding all outgroup sequences. This was done to assess potential effects of unbalanced sampling of ingroup vs. outgroup. Moreover, most substitutions may occur between outgroup and ingroup, rather than within the ingroup, thus risking an inadequate substitutional model for the ingroup, and potentially obscuring ingroup relationships.

2.4.2. Selection of substitution models and partitioning scheme

Appropriate models of substitution were selected for each of the three markers, both while including and excluding outgroup sequences. To select among competing models, we used the ΔAIC score (Posada and Buckley, 2004) calculated by MrModeltest v.2.3 (Nylander, 2004), after obtaining the maximum likelihood under 24 models of sequence evolution and a NJ tree using PAUP* v.4.0b10 (Swofford, 2002). We ignored results for models that incorporate both a proportion of invariable sites (I) and rate variation among sites (modeled with a gamma distribution, Γ), such as GTR + I + Γ, because several initial MCMC runs failed to reach stationarity, likely due to parameter interaction of I and Γ during MCMC (not shown). To avoid under-parameterization (Lemmon and Moriarty, 2004), we considered model selection using AIC to be indecisive when ΔAIC between the best and the second best model was less than 2, and the second best model contained more free parameters.

We then performed Bayesian model selection using BayesFactors instead (Posada and Buckley, 2004). The BayesFactor uses the ratio of the marginal likelihoods of two competing models, taking into account all possible values of the parameters of a model (as well as tree topology) via integration (Brown and Lemmon, 2007). Importantly, BayesFactors can be used to select among non-nested models. We considered the parameter-poor model better fitted and selected it when $2 \times \ln$ BayesFactors > 10 (Brown and Lemmon, 2007). We also used BayesFactors to determine if the cpDNA data was best modeled as one or two partitions (Brown and Lemmon, 2007); the nrDNA data was modeled as a separate partition in all analyses. Similarly, we tested the effect of including and excluding the parameter that describes rate variation among partitions (μ). This prevents the risk of incorrectly using a homogeneous model which has been shown to potentially produce biased topological estimates (Brown and Lemmon, 2007; Brown et al., 2010).

We estimated marginal likelihoods and BayesFactors using Tracer v.1.5.0 (Rambaut and Drummond, 2007), by calculating the harmonic mean of the likelihood scores of post-burnin MrBayes MCMC samples. Although this method for estimating marginal likelihood is most widely used in phylogenetic contexts, other methods (e.g., those based on thermodynamic integration) are known to perform more robustly, but they are computationally substantially more demanding and were therefore not used for the current study (see also Brown and Lemmon, 2007). MrBayes runs for BayesFactors used default priors (unless specified otherwise) for all parameters except for the alpha shape parameter of Γ, where a uniform prior between 0.01 and 50 was used, and consisted of four Metropolis coupled chains (temperature 0.05) that were calculated for 8 million generations, sampling every 1000th generation. Four independent runs per analysis were combined after MCMC diagnosis using Tracer v.1.5.0, making sure that every parameter had converged to the target distribution, and discarding the first 25% of samples of each run as burnin.

2.4.3. Effects of outgroup branch length on branch length prior

Initial exploratory analysis of our data revealed that Bayesian estimates of the total tree length (TL; the sum of the length of all branches in the tree) were several orders of magnitude longer than the maximum likelihood estimate, while topological differences were subtle (not shown). Causes of this "long tree artifact" have been investigated (Yang and Rannala, 2005; Marshall et al., 2006; Brown et al., 2010; Marshall, 2010; Rannala et al., 2012) and warrant scrutiny of the influence of the prior distribution of branch lengths on phylogeny estimation. Branch lengths priors are usually specified with an exponential distribution with mean $1/\lambda$ substitutions per site. Hence, MrBayes' default $\lambda = 10$ corresponds with a prior belief that branches on average have a length of 0.1 substitutions per site. Because our datasets include multiple accessions per species, with little sequence divergence, we expect many branches to be much shorter. Therefore, we tested the influence of the branch length prior on the combined datasets including and excluding outgroup sequences, as advised by Brown et al. (2010). We used values for λ of 1, 10 (default), 20, 50, 100, 200, 1000 for both datasets, and additionally 2000 and 10,000 for the dataset without outgroups. We calculated BayesFactors (as in Section 2.4.2), to determine the optimal λ value when including or excluding outgroup sequences. Because interactions between branch length priors and the μ parameter that describes rate variation among partitions can cause poor MCMC performance (Marshall et al., 2006), we investigated the effect of branch length prior in analyses without μ. However, inclusion of μ for branch length tests in the dataset with outgroups yielded qualitatively identical results (not shown).

2.4.4. Phylogeny estimation

For each of the six datasets, 10 independent MCMC MrBayes runs were calculated for 10 million generations, using default priors unless others were selected as described in Sections 2.4.2 and 2.4.3. MCMC performance and convergence of all runs were checked using Tracer v.1.5.3, after which runs were combined and consensus trees including posterior probabilities of branches were calculated using MrBayes v.3.1.2. For comparison, a parsimony analysis of the concatenated data with outgroup was conducted in PAUP as a full heuristic search with 10 random addition sequence replicates, TBR branch swapping, and MaxTrees set to 10,000.

An alternative approach to outgroup rooting is to infer the phylogeny as an ultrametric, rooted tree by assuming a molecular clock (clock rooting). This was performed in BEAST 1.6.2 (Drummond and Rambaut, 2007), enforcing rate constancy across branches (which is a reasonable assumption given overall low amounts of sequence divergence), but allowing for different substitution rates between the nrDNA and cpDNA markers. On the concatenated dataset without outgroups, we ran 6 independent runs of 10 million generations each, which were combined after confirming convergence using Tracer v.1.5 and discarding 10% burnin. Results were summarized by calculating the maximum clade credibility tree with median node heights.

2.4.5. Genealogical Sorting Index

The Genealogical Sorting Index (GSI, Cummings et al., 2008) provides a measure of the relative degree of exclusive ancestry of a given group of individuals (sequences, samples or OTUs) on a phylogenetic tree, where the maximum value of 1 indicates monophyly and the minimum of 0 indicating dispersal over the entire tree. This relatively simple test uses topological information only, but adequately deals with incompletely resolved relationships. Statistical significance is assessed with a permutation test that generates trees with randomly rearranged individuals. The frequency of GSI values for a group of individuals in the permuted trees that are equal to or greater than the GSI in the original tree provides the *p*-value for rejection of the null hypothesis that the group is of mixed ancestry. The GSI has been used in previous studies to investigate cryptic species and to examine species boundaries (e.g., Cranston et al., 2009; Weisrock et al., 2010; Sakalidis et al., 2011).

The GSI was calculated and permutation tests were conducted using the functions made available on the website www.genealogicalsorting.org. After removal of the outgroup, GSI values were produced for the consensus trees resulting from the combined plastid dataset and the nrITS dataset, and for both trees together (''ensemble statistics''). The permutation tests were run with 10,000 replicates.

2.4.6. Inference of species tree

We employed *BEAST (Heled and Drummond, 2010) implemented in BEAST 1.6.2 (Drummond and Rambaut, 2007) for a Bayesian estimate of the species tree. *BEAST uses the multispecies coalescent and MCMC sampling to calculate the posterior probability of the species tree, using the probability of the data given the gene trees (separately for nrDNA and cpDNA) and that of the gene trees given the species tree. We ran 8 independent runs of 50 million generations each (sampling every 2500th generation), which were combined after confirming convergence using Tracer v.1.5 and excluding 25% burnin. We summarized results by calculating the maximum clade credibility species tree using median node heights.

Species phylogeny was also inferred in Mesquite 2.72 (Maddison and Maddison, 2009) from the cpDNA and nrDNA consensus trees (Figs. 2 and 3) by heuristically searching for the tree that minimizes the number of deep coalescences for multiple loci (Maddison, 1997). Search parameters were set to auto-resolution of contained polytomies, using branch lengths of contained trees, SPR rearrangement, MaxTrees set to 100.

3. Results

3.1. Characteristics of the sequence data

Despite repeated attempts, it was not possible to produce *rps16-trnK* sequences for two ingroup samples (*Primula elatior* #9 and *P. vulgaris* #12), *trnS-trnG* sequences for two ingroup samples (*P. elatior* #14 and *P. vulgaris* #13), and ITS sequences for one outgroup sample (*P. inayatii*). Cloned sequences of the nrITS region for the most part revealed only slight differences (1–4 nucleotide substitutions) between paralogues. A small number of clones contained only parts of the target region, e.g. only the first or only the second half. In preliminary analyses, all cloned sequences from the same specimen clustered together, and did not cause trees to be relevantly different from those based only on sequences from direct PCR (not shown). Because differences between cloned ITS sequences were within the range expected by sequencing error through PCR and phylogenetically inconsequent, we used ITS sequences produced directly from PCR products in most cases. However, in four cases, (*P. elatior* #16 and #18, *P. grandis* #2, *P. juliae* #2), one clone showing a complete ITS sequence was selected to represent the ITS sequence of that sample because the sequences produced directly from PCR products were of inferior quality.

The *rps16-trnK* dataset used for phylogenetic analysis comprised 970 characters, 837 of which were constant, 66 variable but parsimony-uninformative, and 67 variable and parsimony-informative. The *trnS-trnG* dataset comprised 567 characters, 463 of which were constant, 59 variable but parsimony-uninformative, and 45 variable and parsimony-informative. The ITS dataset comprised 666 characters, 466 of which were constant, 88 variable but parsimony-uninformative, and 112 variable and parsimony-informative. It encompassed most of the ITS1, the entire 5.8S rDNA, and the entire ITS2. No recombination was detected in any of the three datasets ($\Phi > 0.05$). We combined the two plastid regions for all subsequent analyses, modeling them as two partitions.

Examination of the phylograms produced for both datasets revealed that discrepancies between gene trees were limited to nodes with very poor statistical support (for example, *P. vulgaris* was metaphyletic, i.e. neither clearly monophyletic nor non-monophyletic, in the plastid tree and monophyletic in the nuclear tree, but with non-significant posterior probability in both cases; Figs. 2 and 3). Therefore we also performed phylogenetic analyses on the combined dataset.

3.2. Selection of substitution models, partitioning scheme and branch length priors for inference of gene trees

Models selected by AIC were GTR + G for the *rps16-trnK* and *trnS-trnG* data including outgroups, GTR + I for the *rps16-trnK* and *trnS-trnG* data excluding outgroups, SYM + I for the ITS data without outgroups, and we performed Bayesian model selection for ITS including outgroups as AIC was inconclusive between SYM + G and GTR + G. The BayesFactor was 1.77, marginally in favor of GTR + G, which was thus selected as it represents the more parameter-rich model (see Supplementary Table 1 for a more inclusive summary). The concatenated datasets with and without outgroups were determined to be best partitioned by region (three partitions), where BayesFactors indicated that analyses with μ were preferred for the dataset with outgroups, and without μ was preferred for the dataset without outgroups (Supplementary Table 2). Branch length priors affected tree length and the estimated marginal likelihood considerably in the dataset including outgroups and to a lesser extent in the dataset without outgroups, particularly when exponential prior distributions had unrealistically high means. Values for λ of 200 and 1000 were selected as most appropriate for the final runs with and without outgroups, respectively. Under these priors, the last significant improvements of marginal likelihood were achieved when progressively decreasing the value of λ (see Supplementary Table 2). It should be noted, however, that the effect of this prior was nearly restricted to the scaling of the tree; tree topology and support of individual clades were virtually the same regardless of branch length prior. See Supplementary Table 3 for a summary of settings used in the final analysis runs.

3.3. Topology

The cpDNA phylogeny shows section *Primula* as clearly monophyletic with a posterior probability of 100%, but its internal structure is partly unresolved (Fig. 2). The only clades that received significant support (i.e. $\geqslant$95%) are small groupings of few samples: two Spanish representatives of *P. elatior* (6, 15); two representatives of *P. megaseifolia* (1, 2); both samples of *P. grandis* included in the study; all three samples of *P. juliae*; all three samples of *P. elatior* ssp. *meyeri*; and a clade of *P. renifolia* and several European samples of *P. elatior* (1, 2, 4, 5, 8, 9, 10, 23).

Fig. 2. Rooted phylogram from Bayesian Posterior Probability Analysis for the concatenated plastid dataset with outgroup. Numbers above the branches are PP values; thick branches indicate PP ⩾ 95%. The inlay illustrates uncut branch lengths.

The nrDNA tree (Fig. 3) also presented section *Primula* as clearly monophyletic and shows more resolution inside the group, although not all clades are significantly supported. Well-supported clades include the three samples of *P. megaseifolia*, the two samples of *P. grandis*, two samples of *P. vulgaris* ssp. *sibthorpii* (4, 6), two samples of *P. elatior* ssp. *meyeri* (16, 18), and a large clade containing part of *P. elatior* and all representatives of *P. veris* except one. Major topological differences between the trees inferred from the separate genomic datasets are found in the non-monophyly of *P. vulgaris* in the cpDNA tree vs. the monophyly of that species in the nrDNA tree, an even less cohesive *P. elatior* in the nuclear tree, and a different position of the root.

3.4. Similarities between plastid and nuclear data

Plastid and nrITS trees agree in several important regards. Results from both chloroplast and nuclear datasets show *P. elatior* as non-monophyletic with regard to most or all other species. Most of its sequences form one clade together with *P. veris*, and in both phylograms there is another clade of several sequences of *P. elatior* that also includes the only sample of *P. renifolia*. *Primula vulgaris* sequences are much less interdigitated with other sequences from other species. Samples of *P. veris* are, with the exception of one sample in the nrITS dataset, restricted to one clade, but strongly intermingled with part of *P. elatior*, and both species share haplo-

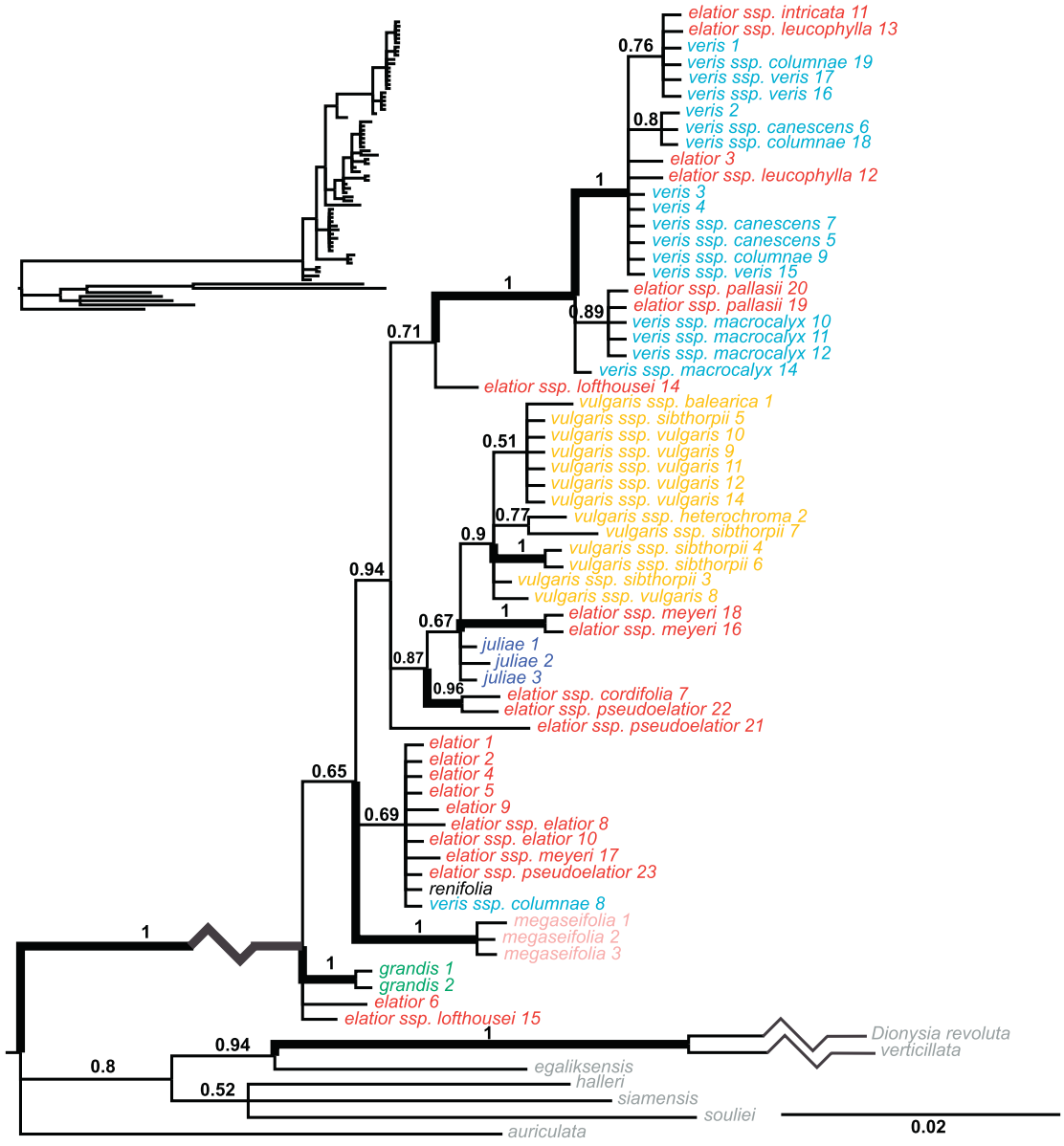

Fig. 3. Rooted phylogram from Bayesian Posterior Probability Analysis for the nrITS dataset with outgroup. Numbers above the branches are PP values; thick branches indicate PP ⩾ 95%. The inlay illustrates uncut branch lengths.

types. Sequences of *P. juliae* group together and close to those of *P. vulgaris*, and the two sequences of *P. grandis* form a small clade in both trees (Figs. 2 and 3).

For the three widely distributed species, our analyses indicate strong genetic heterogeneity for *P. elatior* compared with stronger genetic homogeneity of *P. vulgaris* and *P. veris*, and a close affiliation of *P. veris* with part of *P. elatior*.

3.5. Incongruence between plastid and nuclear data

There are also some apparent discrepancies between the two tree topologies, including the degree of non-monophyly of species and patristic distances between them. However, there are no major disagreements between the topologies of both datasets for which both arrangements receive significant support; instead, they only show varying degrees of resolution. *Primula vulgaris* forms a weakly supported clade in the nrITS tree (Fig. 3), but constitutes a basal, metaphyletic and thus unresolved assemblage of sequences in the plastid tree (Fig. 2). The same is true for *P. megaseifolia*, whose three sequences form a small but strongly supported clade in the nrITS tree (Fig. 3) but are part of the basal assemblage in the plastid tree (Fig. 2). Similarly, while the plastid phylogram supports a larger clade consisting of *P. grandis*, the small *P. renifolia*/*P. elatior* clade and the large *P. veris*/*P. elatior* clade, the nrITS phylogram supports a larger clade consisting of the *P. vulgaris*/*P. juliae* clade and the large *P. veris*/*P. elatior* clade. However, none of these two topologies achieves significant support, although the latter is close with a posterior probability of 0.94.

Likewise, different placements of individual samples in the two phylograms generally have poor support (e.g., *Primula veris* ssp.

columnae #8 and *P. vulgaris* ssp. *vulgaris* #12). Consequently, there are no major differences between the results from plastid and nrITS data that cannot be explained by lack of resolution.

3.6. Analysis of the combined dataset

The majority rule consensus tree resulting from a combined analysis shows a higher degree of resolution and more support for individual clades (Fig. 4). Section *Primula* is again clearly monophyletic with a PP of 100%. Other well-supported clades include *P. grandis*, *P. juliae*, *P. megaseifolia*, *P. elatior* ssp. *meyeri*, a clade of *P. renifolia* and several mostly European samples of *P. elatior*, and a large clade of *P. veris* and *P. elatior* p.p. The monophyly of *P. vulgaris* is uncertain, but it is retrieved as a weakly supported clade together with *P. juliae*. *Primula veris*, *P. renifolia*, *P. grandis* and most of *P. elatior* form a clade, while the exact relationships of the remaining three species and of two Spanish samples of *P. elatior* are unresolved. Cladistic analysis of the concatenated data in PAUP produced 10,000 equally parsimonious trees with a length of 645, a consistency index of 0.795, retention index of 0.890 and rescaled consistency index of 0.708. The topology of the strict consensus tree (not shown) is very similar to the results from Bayesian inference and except in not supporting the large clade encompassing *P. grandis*, *P. renifolia*, *P. veris* and most of *P. elatior*. The phylogram produced with clock rooting in BEAST shows a basal spit between *P. veris*, *P. renifolia*, *P. grandis* and most of *P. elatior* on one side and *P. vulgaris*, *P. juliae*, *P. megaseifolia* and the two Spanish samples of *P. elatior* on the other side (not shown), a root position that is equivalent to that resulting from outgroup rooting.

3.7. Genelogical Sorting Index

An overview of GSI values and levels of significance for all species is available in Table 1, except *Primula renifolia*, for which GSI cannot be calculated due to lack of replicate samples. For the combined plastid dataset, permutation tests resulted in rejection of the null-hypothesis that the groups are of mixed ancestry (all $p < 0.001$) for all species except *P. elatior*. However, the GSI was low for *P. megaseifolia* and *P. vulgaris*, indicating a low degree of exclusive genealogical ancestry, and p-values were not highly significant. For both the nrITS dataset and the ensemble of nuclear and plastid trees, all six species achieved highly significant p-values, indicating that the null hypothesis could be rejected for all species, although some species show comparatively low GSI, especially *P. elatior* and *P. veris*.

3.8. Species tree

The species tree inferred by *BEAST consists of two clades, albeit with low support, one comprising *P. juliae*, *P. vulgaris* and *P. megaseifolia*, the latter two as sister species, and the other comprising *P. grandis*, *P. renifolia* and the sister species *P. elatior* and *P. veris* (Fig. 5A). The search for a species phylogeny minimizing deep coalescences produced six best trees with a score of 69 deep coalescences (25 from the contained nrITS tree, 44 from the plastid tree) differing only in the topology of the outgroup. In this reconstruction, the same two clades are recovered but *P. juliae* and *P. megaseifolia* are sister species, as are *P. grandis* and *P. renifolia* (Fig. 5B).

4. Discussion

The present study explored problems arising from the combination of phylogenetic isolation and lack of genetic differentiation among taxonomically distinct species. Our analyses, based on complete taxon sampling at the subspecific level, broad geographic sampling, and a combination of methodological approaches, revealed striking non-monophyly of sequence in some of several widespread but morphologically well-characterized species (Figs. 2–4). It also serves as a case study to examine the effect of the choice of branch length prior for Bayesian phylogenetic analyses of isolated ingroups.

4.1. Non-monophyly of haplotypes in species

Reciprocal non-monophyly and sharing of haplotypes between sister species is an expected transitional stage for diverging lineages (Tajima, 1983; Avise and Ball, 1990; Maddison, 1997), unless a species diverges through a severe population bottleneck. Even then, haplotypes in the parental species would at first be paraphyletic to those in the newly established population. Empirical data suggest that mitochondrial haplotypes are non-monophyletic in ca. 23% of animal species (Funk and Omland, 2003). While a similar assessment seems to be unavailable for plants, Crisp and Chandler (1996) concluded that 21% of the Australian plant species they examined were potentially paraphyletic based on morphological characters. Over time, genetic drift will progressively lead to lineage sorting through the extinction of haplotype families in each species, until haplotypes in sister species become reciprocally monophyletic. The likelihood of achieving this exclusive ancestry after a given time is influenced by effective population size (Hudson and Coyne, 2002).

Despite this, the observation of non-monophyly has in recent years prompted taxonomic reassessments or the formal segregation of para-species into separate cryptic species (e.g., Cranston et al., 2009; Carstens and Dewey, 2010; Weisrock et al., 2010; Sakalidis et al., 2011). These taxonomic approaches are motivated by the desire to identify independent and exclusive lineages as subjects of evolution and preferred targets of conservation strategies.

Sequences from all species of which more than two samples were included in the present study are at least metaphyletic if not paraphyletic for at least one of the two datasets. The most complicated case is presented by *Primula elatior*. Its plastid sequences are, if only significantly supported clades are taken into account, paraphyletic to *P. renifolia* and metaphyletic to all other species (Fig. 2). Its nrITS sequences are paraphyletic to those from *P. veris* and metaphyletic relative to all other species (Fig. 3). In the total evidence analysis, *P. elatior* is paraphyletic to *P. grandis*, *P. renifolia* and *P. veris*, and metaphyletic relative to the remaining three species (Fig. 4).

This situation is perhaps unsurprising, as *P. elatior* is also the morphologically most diverse of the currently accepted species, and has been divided into the largest number of subspecies and formerly segregate species (Komarov, 1963; Richards, 2003; Kovtonyuk and Goncharov, 2009). Unfortunately, neither subspecific affiliation nor geographic provenance provide useful clues to the underlying reasons for the topology of the phylograms. In the absence of discernible morphological or geographic structure, there is consequently at present no obvious way of dividing *P. elatior* into meaningful lineages that would help explain the origin of the observed non-monophyly of the sequences found in the species in terms of a wrong taxonomy (McKay and Zink, 2010) currently being applied (see also Section 4.3 below), even if it were entirely uncontroversial what "wrong taxonomy" even means and, in particular, if the criterion of monophyly can meaningfully be applied to species (Mishler, 1999; Rieppel, 2010).

The remaining explanations for non-monophyly of sequences in a species are ancestral polymorphism and recent introgression of haplotypes from another species (Wendel and Doyle, 1998). Results from the permutation test for significance of the GSI in the

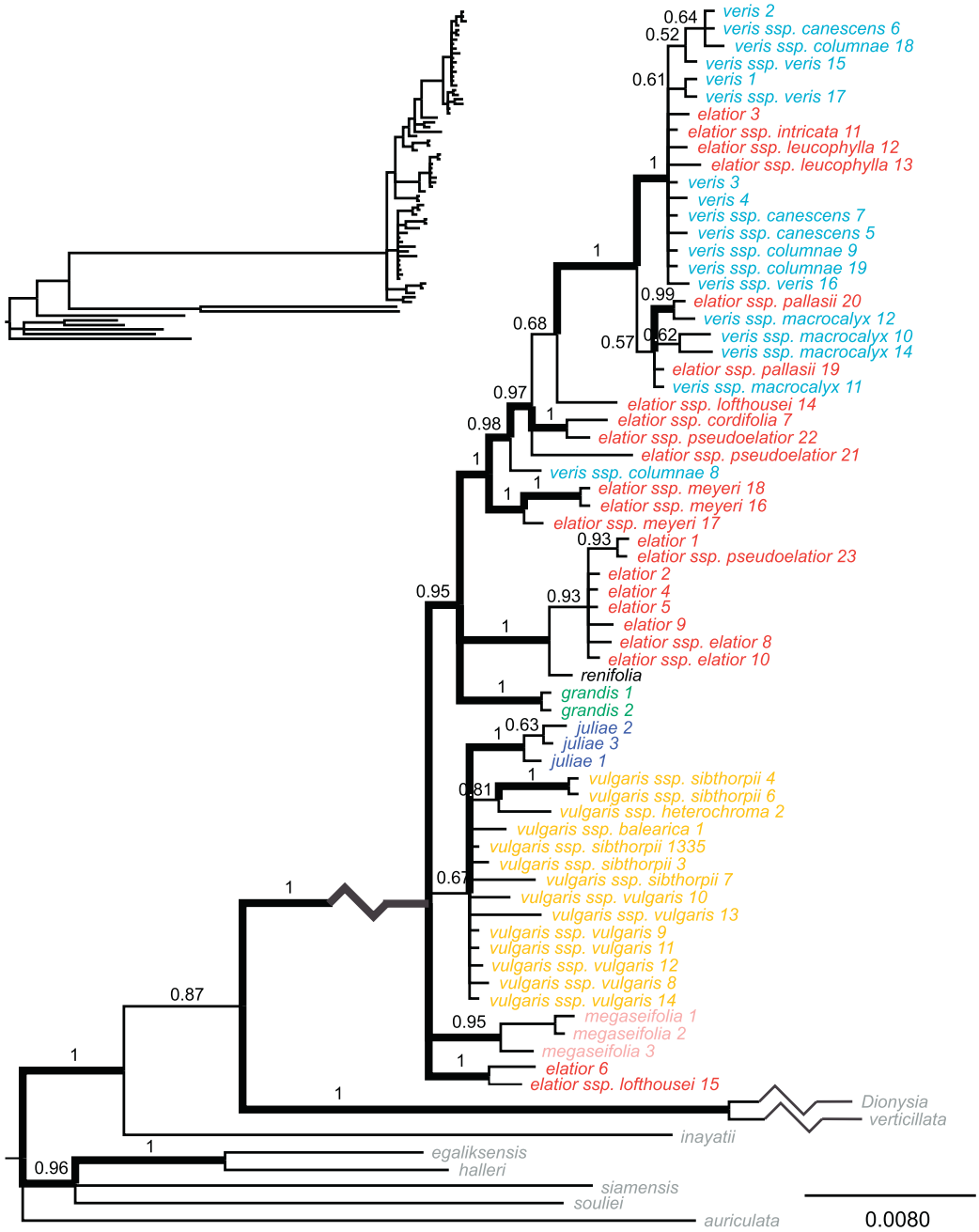

Fig. 4. Rooted phylogram from Bayesian Posterior Probability Analysis for the concatenated dataset with outgroup. Numbers above the branches are PP values; thick branches indicate PP ⩾ 95%. The inlay illustrates uncut branch lengths.

ensemble dataset indicate that the null hypothesis of mixed ancestry can be rejected for *P. elatior* despite the non-monophyly of its sequences relative to other species, failure of the GSI test for the plastid dataset alone and overall fairly low GSI values (Table 1). This would imply that our results are compatible with the assumption that the species is forming an independent lineage and not significantly introgressing with the other species. It should be noted that *P. elatior* supplied the largest number of samples of all species to our study, and the probability of observing significant GSI values actually decreases with increasing group size (Cummings et al., 2008).

If all samples of *P. elatior* were removed from the phylograms, levels of non-monophyly would decrease markedly: All other species except *P. vulgaris* and *P. megaseifolia* would be monophyletic on the plastid tree, all except *P. veris* on the nrITS tree, and all species would be metaphyletic or monophyletic on the tree from concatenated analysis. This could be interpreted as additional support for ancestral polymorphism as the main cause of non-monophyly,

Table 1
Genealogical Sorting Index (values) and significance levels (symbols) for rejection of the null-hypothesis of mixed ancestry. n.s. indicates non-significant. *Primula renifolia* is represented with only one sample in our datasets, and therefore, no GSI values could be calculated.

Species	nrITS	Combined plastid	Both genomes
Primula elatior	0.207***	0.067 (n.s.)	0.137***
P. grandis	1.000***	1.000***	1.000***
P. juliae	0.483***	1.000***	0.742***
P. megaseifolia	1.000***	0.089*	0.544***
P. veris	0.255***	0.462***	0.359***
P. vulgaris	1.000***	0.096*	0.548***

* $p < 0.05$.
*** $p < 0.001$.

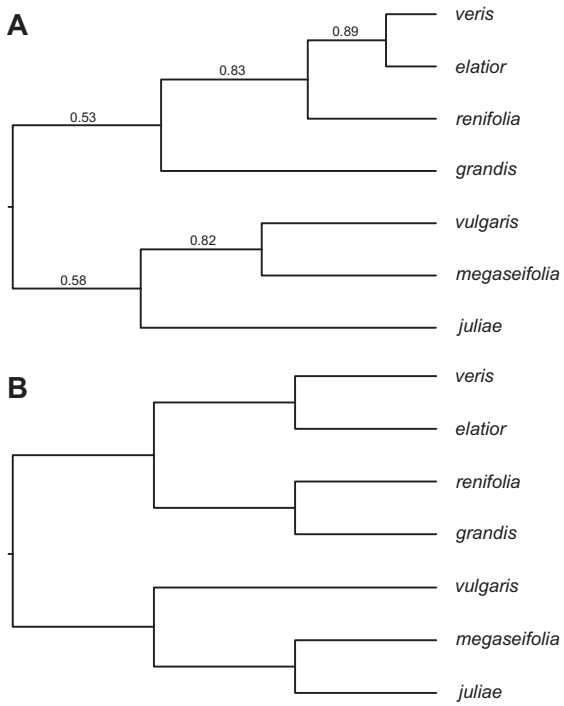

Fig. 5. Species tree of section *Primula* as inferred by a *BEAST analysis (A) and minimizing deep coalescences (B). Numbers above branches in A are Bayesian posterior probabilities.

with *P. elatior* as the paraphyletic remnant of the sections's ancestral species and all other species as genetically more homogeneous recent segregates.

On the other hand, *P. elatior*, *P. veris* and *P. vulgaris* are known to hybridize frequently (Heslop Harrison, 1931; Smith et al., 1984; Gurney et al., 2007). Although the hybrids often show reduced fertility (Valentine, 1952, 1955), it is currently unclear to what degree they backcross into the parental species, and the two species that are most commingled genetically, *P. veris* and *P. elatior*, are also the two that are most difficult to cross of the three extra-Caucasian species (Valentine, 1952), introgression and chloroplast capture might be expected. From this perspective, sequences from *P. elatior* found in the clades containing *P. veris* and *P. vulgaris* could have introgressed from those species. This interpretation would suggest a biased flow of genes between species, as, for example, not much introgression would appear to have taken place into *P. vulgaris* (but see Fig. 2 for one exception). Alternatively, the signature of introgression may be obscured through concerted evolution of nrITS sequences (Hamby and Zimmer, 1992).

Based on our data, it is thus not possible to decide whether the rampant non-monophyly of *P. elatior* is due to hybridization, ancestral polymorphism, or both processes, although the ensemble GSI indicates that the results would be compatible with ancestral polymorphism. Future studies using genomic data or population genetic approaches may help to address the issue more decisively.

4.2. Phylogeny of the section

As in previous studies (Mast et al., 2001, 2006; Kovtonyuk and Goncharov, 2009), gene trees (Figs. 2 and 3) as well as the tree produced from the concatenated dataset (Fig. 4) show a section *Primula* including *P. grandis* as a clade situated on a very long branch, but with very short internal branches. While the other species are comparatively similar to each other morphologically, this situation is more surprising for *P. grandis* with its unusual occurrence of farina and its homostylous, pendent flowers, suggesting fast evolution of characters in that species, perhaps in relation to a shift in pollination syndrome.

A clear resolution of the phylogeny of section *Primula* is hampered by the non-monophyly of *P. elatior* in relation to all other species, discrepancies between nuclear and plastid data, and, in particular, rooting problems arising from the combination of low sequence divergence within the section and its very strong divergence from the outgroup. This latter problem expresses itself in the recovery of basal polytomies from phylogenetic analyses (Fig. 4). Nevertheless, under the caveat of these uncertainties, both the phylogram based on the concatenated datasets (Fig. 4) and the species trees (Fig. 5) can yield some insights.

Perhaps the taxonomically most surprising result is that *P. renifolia* does not seem to be closely related to *P. megaseifolia*, as suggested by Komarov (1963). Smith and Fletcher (1948) even considered the plants to be conspecific. Instead, the only sample of *P. renifolia* included in this study falls into a clade of Caucasian to European samples of *P. elatior*, which is in turn part of a larger clade containing all of *P. veris*, *P. grandis* and most of *P. elatior* (Fig. 4). These four species are also grouped into one clade in the species trees, which also show *P. elatior* and *P. veris* as sister species (Fig. 5). This suggested relationship comes as no surprise under the assumption of no hybridization that underlies available species tree methods (e.g., Maddison, 1997), as virtually all samples of *P. veris* and the majority of samples of *P. elatior* are found in one clade in both gene trees (Figs. 2 and 3), implying a very recent divergence of the former species with unfinished lineage sorting.

The remaining three species (*P. juliae*, *P. megasaeifolia*, *P. vulgaris*) may be closely related to each other as indicated by the species trees (Fig. 5), but the gene trees and the phylogram from the total evidence analysis (Fig. 4) are ambiguous on their position and place them on polytomies or in very weakly supported clades. A final assessment will have to be withheld until additional data are available. A sister group relationship of *P. juliae* and *P. vulgaris*, as suggested by the phylogram based on the concatenated datasets (Fig. 4) but not by the species trees (Fig. 5), would, however, make sense from a morphological perspective, as they are the only species in the section that do not produce their flowers in umbels – *P. renifolia* at first sight also appears to have solitary flowers, but according to Richards (2003) the scape is merely very short and elongates when the plant is fruiting.

4.3. Taxonomic considerations

The unambiguous position of *P. grandis* among the species of section *Primula* in all molecular analyses suggests that, despite the striking morphological differences, recognition of a separate,

monotypic section *Sredinskya*, as still found in Richards (2003), is untenable, because it would most likely make remaining section *Primula* paraphyletic.

In those cases where multiple samples of the various subspecies of *P. veris*, *P. vulgaris* and *P. elatior* were available, they generally do not show any apparent genetic differentiation from each other (e.g., see the position of the representatives of *P. elatior* ssp. *pseudoelatior* and ssp. *pseudoelatior* in Fig. 4). It is thus unlikely that any of the current subspecies are better treated as segregate species, e.g. *P. macrocalyx* Bunge or *P. amoena* M. Bieb., as the sequence data available in the present study does not give any indication of them forming independent lineages.

On the other hand, sequences from *P. elatior* fall into different clades in all trees, with most of the affiliations of the individual samples congruent between nrITS and plastid analyses: most sequences fall into a clade together with *P. veris*, which is spread over all of temperate Eurasia; a smaller group dominates the clade containing *P. renifolia*, found only in samples from Western Europe to the Caucasus, and the remaining few, from Spanish and Caucasian samples, are scattered over the rest of the phylograms (Figs. 2–4). Some taxonomists advocate the formal recognition of every monophyletic group of individuals (if this term can be applied within species in the first place; i.e. effectively of every independently evolving lineage), no matter how morphologically cryptic (e.g., Mishler, 1999; Sakalidis et al., 2011), and would perhaps suggest assigning some taxonomic status to the groups of individuals of *P. elatior* appearing in separate clades together with *P. veris* or *P. renifolia*, respectively. Unfortunately, not only are the clade affiliations of sequences in this case completely at odds with subspecies affiliation and thus to the distribution of many morphological characters that can be assumed to also be evidence of relatedness, but such a solution would also leave behind a remnant of disparate individuals not belonging to either clade. It thus seems preferable to maintain a broad circumscription of *P. elatior* while acknowledging its much larger genetic and morphological heterogeneity in comparison to the other species of the section, at least until a more extensive study can be conducted.

4.4. Branch length priors

Phylogenetic analyses were complicated by the combination of low sequence divergence in the ingroup and a high degree of isolation from any outgroup sample, leading to difficulties with the recovery of realistic branch lengths (Brown et al., 2010; Marshall, 2010; Rannala et al., 2012). This problem is of most concern when absolute branch lengths or absolute mutation rates are of interest (Brown et al., 2010). For our dataset, the degree of sequence divergence, measured by branch lengths in expected number of substitutions per site, was greatly affected by the prior on branch lengths (Supplementary Table 2). Default MrBayes branch length priors would have resulted in an overestimation of overall sequence divergence of orders of magnitude. These results illustrate that incorrectly specified priors can heavily bias the observed amount of phylogenetic differentiation among taxa, warranting careful specification of priors (Rannala et al., 2012).

5. Conclusions

With *Primula elatior*, *Primula* sect. *Primula* presents a particularly striking example of species non-monophyly, in that the species is non-monophyletic with regard to most, if not all, others of its section. Three possible explanations of species non-monophyly are usually suggested: ancestral polymorphism, incomplete reproductive isolation and 'wrong' taxonomy. Because the Genealogical Sorting Index for this species is significant in the ensemble analysis, and because there is no apparent geographic or morphological signal in its genetic structure, it is possible that introgression plays only a minor role and that *P. elatior* as currently circumscribed is the disjointed remnant of the ancestral species of the entire section. On the other hand, available data do not permit to decisively rule out a greater role for introgression, and it could also be argued that the diverse clade affiliations of samples from *P. elatior* reflect introgression events from other species. It is interesting to note that the species' genetic heterogeneity is mirrored by its morphological diversity and complicated taxonomic history. However, currently no convincing alternative to the treatment as one species presents itself, and the other species of the section are morphologically much more divergent than the current subspecies of *P. elatior*. After removal of all samples of *P. elatior*, no other species of section *Primula* would be strongly non-monophyletic, although levels of resolution differ between nuclear and plastid datasets.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.05.015.

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Supplementary Table 1. Results of selection of substitution models based on AIC. Underlined models were selected for final analyses.

Dataset	Model	Negative log likelihood	Number of estimated (free) parameters, K	AIC score	AIC difference
nrITS Incl. outgroups	SYM+G	2573.04	6	5158.07	0.00
	GTR+G	2570.60	9	5159.19	1.12
	SYM+I+G	2572.93	7	5159.86	1.79
	GTR+I+G	2570.49	10	5160.98	2.91
	SYM+I	2575.30	6	5162.59	4.52
	GTR+I	2572.73	9	5163.45	5.38
	HKY+G	2585.75	5	5181.49	23.42
	HKY+I+G	2585.60	6	5183.20	25.13
	HKY+I	2588.67	5	5187.35	29.28
	K80+G	2591.83	2	5187.67	29.60
	K80+I+G	2591.69	3	5189.37	31.30
	K80+I	2594.82	2	5193.65	35.58
	SYM	2626.55	5	5263.10	105.03
	GTR	2624.25	8	5264.50	106.43
	HKY	2639.53	4	5287.05	128.98
	K80	2646.87	1	5295.74	137.67
	F81+G	2651.41	4	5310.81	152.74
	F81+I+G	2651.28	5	5312.57	154.50
	F81+I	2654.45	4	5316.91	158.83
	JC+G	2659.69	1	5321.38	163.30
	JC+I+G	2659.59	2	5323.19	165.12
	JC+I	2662.48	1	5326.97	168.90
	F81	2705.22	3	5416.44	258.36
	JC	2712.69	0	5425.39	267.32
***rps16-trnK* Incl. outgroups**	GTR+G	2310.92	9	4639.83	0.00
	GTR+I	2311.62	9	4641.24	1.41
	GTR+I+G	2310.89	10	4641.78	1.95
	GTR	2323.83	8	4663.66	23.82
	HKY+G	2343.59	5	4697.18	57.35
	HKY+I	2344.19	5	4698.39	58.55
	HKY+I+G	2343.51	6	4699.02	59.19
	HKY	2358.91	4	4725.82	85.98
	F81+G	2363.77	4	4735.53	95.70
	F81+I	2364.16	4	4736.33	96.50
	F81+I+G	2363.70	5	4737.39	97.56
	F81	2381.94	3	4769.89	130.05
	SYM+G	2459.35	6	4930.71	290.87
	SYM+I	2459.55	6	4931.10	291.26
	SYM+I+G	2459.31	7	4932.62	292.79
	K80+G	2477.21	2	4958.42	318.59
	K80+I	2477.46	2	4958.93	319.09
	K80+I+G	2477.12	3	4960.24	320.40
	SYM	2481.55	5	4973.10	333.27
	JC+G	2490.74	1	4983.47	343.64
	JC+I	2490.98	1	4983.96	344.12
	JC+I+G	2490.65	2	4985.30	345.47
	K80	2499.94	1	5001.89	362.05
	JC	2513.48	0	5026.96	387.12
***trnG-trnS* Incl. outgroups**	GTR+G	1460.40	9	2938.81	0.00
	GTR+I	1461.18	9	2940.35	1.55
	GTR+I+G	1460.40	10	2940.80	1.99
	GTR	1465.88	8	2947.77	8.96
	HKY+G	1479.84	5	2969.68	30.87
	HKY+I	1480.46	5	2970.92	32.11
	F81+G	1481.65	4	2971.29	32.49
	HKY+I+G	1479.83	6	2971.65	32.85
	F81+I	1482.19	4	2972.38	33.58
	F81+I+G	1481.63	5	2973.26	34.46
	F81	1486.98	3	2979.95	41.15
	HKY	1486.05	4	2980.11	41.30

	Model	Negative log likelihood	Number of estimated (free) parameters, K	AIC score	AIC difference
	SYM+G	1533.64	6	3079.27	140.46
	SYM+I	1534.13	6	3080.26	141.45
	SYM+I+G	1533.59	7	3081.18	142.37
	JC+G	1545.98	1	3093.97	155.16
	SYM	1542.26	5	3094.51	155.70
	K80+G	1545.38	2	3094.76	155.96
	JC+I	1546.49	1	3094.98	156.17
	K80+I	1545.91	2	3095.82	157.01
	JC+I+G	1545.96	2	3095.91	157.10
	K80+I+G	1545.35	3	3096.70	157.90
	JC	1553.88	0	3107.75	168.95
	K80	1553.31	1	3108.63	169.82
nrITS Excl. outgroups	SYM+I	1392.88	6	2797.76	0.00
	K80+I	1397.18	2	2798.36	0.60
	SYM+G	1393.25	6	2798.49	0.73
	HKY+I	1394.50	5	2799.01	1.25
	K80+G	1397.64	2	2799.28	1.52
	SYM+I+G	1392.88	7	2799.76	2.00
	HKY+G	1394.96	5	2799.92	2.16
	GTR+I	1391.07	9	2800.13	2.37
	K80+I+G	1397.18	3	2800.36	2.60
	GTR+G	1391.42	9	2800.85	3.09
	HKY+I+G	1394.50	6	2801.01	3.25
	GTR+I+G	1391.07	10	2802.13	4.37
	SYM	1405.91	5	2821.81	24.05
	K80	1410.42	1	2822.84	25.08
	HKY	1407.71	4	2823.42	25.66
	GTR	1404.00	8	2823.99	26.23
	JC+I	1411.32	1	2824.63	26.87
	F81+I	1408.59	4	2825.18	27.42
	JC+G	1411.74	1	2825.47	27.71
	F81+G	1409.03	4	2826.05	28.29
	JC+I+G	1411.32	2	2826.63	28.87
	F81+I+G	1408.59	5	2827.18	29.42
	JC	1424.41	0	2848.83	51.07
	F81	1421.72	3	2849.44	51.68
***rps16-trnK* Excl. outgroups**	GTR+I	1460.90	9	2939.79	0.00
	GTR+I+G	1460.46	10	2940.92	1.13
	GTR+G	1461.54	9	2941.08	1.28
	HKY+I	1471.16	5	2952.33	12.53
	HKY+I+G	1470.64	6	2953.27	13.48
	HKY+G	1472.79	5	2955.58	15.78
	GTR	1470.06	8	2956.12	16.32
	F81+I	1474.97	4	2957.93	18.14
	F81+I+G	1474.46	5	2958.92	19.13
	F81+G	1477.11	4	2962.22	22.43
	HKY	1482.16	4	2972.32	32.53
	F81	1486.88	3	2979.77	39.97
	SYM+I	1581.60	6	3175.19	235.40
	SYM+I+G	1581.13	7	3176.27	236.47
	K80+I	1586.29	2	3176.57	236.78
	K80+I+G	1585.76	3	3177.51	237.72
	JC+I	1588.88	1	3179.76	239.96
	SYM+G	1583.92	6	3179.85	240.05
	JC+I+G	1588.36	2	3180.71	240.92
	K80+G	1589.62	2	3183.23	243.44
	JC+G	1592.21	1	3186.41	246.62
	SYM	1593.71	5	3197.42	257.63
	K80	1600.09	1	3202.18	262.38
	JC	1602.68	0	3205.36	265.57

Dataset	Model	Negative log likelihood	Number of estimated (free) parameters, K	AIC score	AIC difference
trnG-trnS **Excl. outgroups**	GTR+I	904.20	9	1826.39	0.00
	GTR+I+G	905.18	10	1830.36	3.97
	GTR+G	906.20	9	1830.40	4.01
	F81+I	911.82	4	1831.64	5.25
	F81+I+G	911.76	5	1833.52	7.13
	HKY+I	911.81	5	1833.62	7.23
	HKY+I+G	911.75	6	1835.50	9.10
	F81+G	913.75	4	1835.51	9.12
	HKY+G	913.73	5	1837.47	11.08
	GTR	912.19	8	1840.38	13.98
	F81	919.41	3	1844.81	18.42
	HKY	920.57	4	1849.13	22.74
	SYM+I	968.05	6	1948.11	121.71
	SYM+I+G	968.05	7	1950.11	123.71
	JC+I	974.45	1	1950.91	124.52
	SYM+G	970.30	6	1952.59	126.20
	K80+I	974.41	2	1952.81	126.42
	JC+I+G	974.45	2	1952.89	126.50
	K80+I+G	974.40	3	1954.80	128.41
	JC+G	976.96	1	1955.92	129.52
	K80+G	976.91	2	1957.81	131.42
	SYM	977.26	5	1964.52	138.13
	JC	984.08	0	1968.15	141.76
	K80	984.02	1	1970.04	143.64

Supplementary Table 2. Bayesian analyses to select appropriate substitution models, partitioning schemes and branch length priors.

Objective	**Dataset**		**Analysis settings (2)**			**Results**			**Decision**
	Genomic regions (1)	Including outgroup taxa?	Partitioning scheme	Prior on branch lengths, λ (3)	Modeling substitution rate variation among partitions, μ?	Marginal likelihood (4)	Mean tree length	BF support relative to best model (5)	
Selection of substitution model	nrITS, GTR+G model	yes	---	10	---	-2882.67 +/- 0.221	12.54	0.00	GTR+G model selected
	nrITS, SYM+G model	yes	---	10	---	-2883.55 +/- 0.295	12.52	-1.77	
Selection of appropriate partitioning scheme	all	no	by gene	10	no	-4103.24 +/- 0.344	0.23	0.00	Partitioning scheme for dataset without outgroups: by gene (three partitions), no μ
	all	no	by genome	10	no	-4111.77 +/- 0.436	0.23	-17.07	
	all	no	by genome	10	yes	-4117.48 +/- 0.460	0.26	-28.48	
	all	no	by gene	10	yes	-4211.16 +/- 0.450	0.62	-215.84	
Selection of appropriate partitioning scheme	all	yes	by gene	10	yes	-6841.28 +/- 0.391	13.82	0.00	Partitioning scheme for dataset with outgroups: by gene (three partitions), including μ
	all	yes	by genome	10	yes	-6881.25 +/- 0.474	13.01	-79.96	
	all	yes	by gene	10	no	-7267.67 +/- 0.293	12.04	-852.78	
	all	yes	by genome	10	no	-7270.38 +/- 0.248	11.85	-858.20	
Exploration of effect of branch length prior on phylogeny inference and selection of appropriate setting	all	yes	by gene	1	no	-7291.24 +/- 0.269	138.88	-1117.92	Appropriate branch length prior for datasets that include outgroups: λ=200 (3)
	all	yes	by gene	10	no	-7268.12 +/- 0.305	12.01	-1071.68	
	all	yes	by gene	20	no	-6753.68 +/- 0.322	0.53	-42.80	
	all	yes	by gene	50	no	-6747.30 +/- 0.287	0.51	-30.03	

	Genomic regions (1)	Including outgroup taxa?	Partitioning scheme	Prior on branch lengths, λ (3)	Modeling substitution rate variation among partitions, μ?	Marginal likelihood (4)	Mean tree length	BF support relative to best model (5)	
	all	yes	by gene	100	no	-6740.44 +/- 0.305	0.47	-16.31	
	all	yes	by gene	200	no	-6732.28 +/- 0.269	0.42	0.00	
	all	yes	by gene	1000	no	-6755.14 +/- 0.249	0.26	-45.71	
Exploration of effect of branch length prior on phylogeny inference and selection of appropriate setting	all	no	by gene	1	no	-4105.41 +/- 0.347	0.23	-162.34	Appropriate branch length prior for datasets that exclude outgroups: λ=1000 (3), because this value represents the last reduction of prior mean relative to default (λ=10) that significantly improves model fit
	all	no	by gene	10	no	-4103.08 +/- 0.343	0.23	-157.67	
	all	no	by gene	20	no	-4100.67 +/- 0.419	0.22	-152.85	
	all	no	by gene	50	no	-4092.63 +/- 0.353	0.21	-136.77	
	all	no	by gene	100	no	-4082.46 +/- 0.314	0.20	-116.45	
	all	no	by gene	200	no	-4067.13 +/- 0.271	0.17	-85.78	
	all	no	by gene	1000	no	-4028.05 +/- 0.231	0.10	-7.62	
	all	no	by gene	2000	no	-4024.24 +/- 0.205	0.07	0.00	
	all	no	by gene	10000	no	-4086.84 +/- 0.230	0.02	-125.20	

Notes:

1: The three regions used in this study are the nuclear ribosomal internal transcribed spacer (nrITS), and plastid spacer regions *rps16-trnK* and *trnG-trnS*.

2: Analyses were run in MrBayes v.3.1.2, employing four Markov Chain Monte Carlo runs of four metropolis-coupled chains for eight million generations, with temperature 0.05 to ensure proper mixing across parameter space.

3: Prior distributions for branch lengths are exponential, with mean 1/λ, where λ=10 represents the default value in MrBayes v.3.1.2.

4: Marginal likelihood is calculated using the harmonic mean estimator implemented in Tracer v.1.5, with standard error obtained via 100 bootstraps

5: BayesFactor (BF) relative support for model i is calculated as the 2 times the difference in log likelihood between the best model and model i, where 10 is considered significant, following Brown and Lemmon (2007)

Supplementary Table 3. Settings of final Bayesian analyses.

Dataset		**Settings for final analyses**						
Genomic regions (1)	Including outgroup taxa?	Partitioning scheme (2)	Modeling substitutional rate variation among partitions, μ? (2)	Prior on branch lengths, λ (2,3)	Number of independent runs per analysis	Number of metropolis-coupled chains per run	Temperature	Number of generations per chain
all	yes	by gene	yes	200	12	4	0.05	10 million
cpDNA	yes	by gene	yes	200	10	4	0.05	10 million
nrITS	yes	---	---	200	10	4	0.05	10 million
all	no	by gene	no	1000	10	4	0.05	10 million
cpDNA	no	by gene	no	1000	12	4	0.05	10 million
nrITS	no	---	---	1000	12	4	0.05	10 million

Notes:

1: The three regions used in this study are the nuclear ribosomal internal transcribed spacer (nrITS), and plastid spacer regions *rps16-trnK* and *trnG-trnS*.

2: Setting determined through a series of preliminary runs, see Supplementary table 2

3: Prior distributions for branch lengths are exponential, with mean 1/λ.

Appendix. List of samples used in the present study, voucher information and Genbank accession numbers.

Sample ID	Taxon	Provenance	Voucher	nrITS	rps16-trnK	trnS-trnG
1	*Primula elatior* (L.) Hill	Switzerland, cultivated at the Botanical Garden of Zurich, #20051293, originally from Slovenia, Julian Alps	Schmidt-Lebuhn 1012 (Z)	HM629066	HM628912	HM628978
2	*Primula elatior* (L.) Hill	Switzerland, cultivated at the Botanical Garden of Zurich, #20051346, originally from Slovakia, Vysoké Tatry, Slanske Vrchy	no voucher	HM629067	HM628913	HM628983
3	*Primula elatior* (L.) Hill	Switzerland, cultivated at the Botanical Garden of Zurich, #20080828, originally from Denmark, Bornholm, Skelbro, Risebaek	Ketelhut 15 (B)	HM629071	HM628914	HM628984
4	*Primula elatior* (L.) Hill	Switzerland, cultivated at the Botanical Garden of Zurich, #20090112, originally from Wallis, Sion, W-side of Lac de Tseuzier, raised from seeds obtained from Hortus Botanicus Reykjavicensis, Iceland	no voucher	HM629085	HM628915	HM628985
5	*Primula elatior* (L.) Hill	Poland, Ogród Botaniczny UMCS-LUBLIN 11-Polen from Tatry, Grzybowiec 1800 m	Photo Voucher Michał Czernecki s.n. (Z)	HM629073	HM628916	HM628986
6	*Primula elatior* (L.) Hill	Spain, Avila, Sierra de Gredos, puerto de Peña Negra, c. 1500 m, 8 V 2009	Vargas 3PV09 (Z)	HM629102	HM628917	HM628987
7	*Primula elatior* subsp. *cordifolia* (Rupr.) Smith & Forrest	United Kingdom, private cultivar collection of Ian Scott, 2000	no voucher	HM629132	HM628918	HM628989
8	*Primula elatior* (L.) Hill subsp. *elatior*	Cultivated by Ron McBeath, Lamberton Nusery, Berwickshire, Scotland, 2000; originally from East Anglia	Mast 722 (Z)	HM629098	HM628919	HM628990
9	*Primula elatior* (L.) Hill subsp. *elatior*	Switzerland, cultivated at the Botanical Garden of Zurich, #19780106	Mast 425 (Z)	HM629106	none	HM628988
10	*Primula elatior* (L.) Hill subsp. *elatior*	Cultivated by Ron McBeath, Lamberton Nusery, Berwickshire, Scotland, 2002; originally from East Anglia	no voucher	HM629130	HM628920	HM628991
11	*Primula elatior* subsp. *intricata* (Gren. & Godr.) Ludi	Switzerland, cultivated at the Botanical Garden of Zurich, #20051460, originally from Italia, Valle di Palombaro, Palombaro	Schmidt-Lebuhn 1274 (Z)	HM629068	HM628921	HM628992
12	*Primula elatior* subsp. *leucophylla* (Pax) Heslop-Harrison	Switzerland, cultivated at the Botanical Garden of Zurich, #20050874, raised from seeds obtained from University of Iasi, Romania	no voucher	HM629065	HM628922	HM628993
13	*Primula elatior* subsp. *leucophylla* (Pax) J. Heslop-Harrison	Switzerland, cultivated at the Botanical Garden of Zurich, #20090125, raised from seeds obtained from University of Iasi, Romania	no voucher	HM629072	HM628923	HM628994

14	*Primula elatior* subsp. *lofthousei* (J. Heslop-Harrison) Smith & Fletcher	Spain, Holla Mora (Sierra Nevada), 2300 m, 6 Jun 1971	Anonymous s.n. (BCN #11842)	HM629160	HM628924	none
15	*Primula elatior* subsp. *lofthousei* (J. Heslop-Harrison) Smith & Fletcher	Spain, HS Granada, Sierra Nevada	WG 37221 (Z)	HM629188	HM628925	HM628995
16	*Primula elatior* subsp. *meyeri* (Rupr.) Valentine & Lamond	United Kingdom, private cultivar collection of John Mattingley, 2000	no voucher	HM629124	HM628927	HM628997
17	*Primula elatior* subsp. *meyeri* (Rupr.) Valentine & Lamond	Cultivated by Ron McBeath, Lamberton Nusery, Berwickshire, Scotland, 2000, originally from Georgia	no voucher	HM629131	HM628928	HM628998
18	*Primula elatior* subsp. *meyeri* (Rupr.) Valentine & Lamond	Georgia, Mtskheta-Mtianeti, Great Caucasus, from church Tsminda Sameba in direction of Mt. Kasbek (44°33'05" E, 42°39'34" N), 2650-3160 m, 19 Jul 2002	Schneeweiss 8672 (WU, not seen)	HM629116	HM628926	HM628996
19	*Primula elatior* subsp. *pallasii* (Lehm.) Smith & Forrest	Cultivated by Ron McBeath, Lamberton Nusery, Berwickshire, Scotland, 2000, “JJ 256”	no voucher	HM629133	HM628930	HM629000
20	*Primula elatior* subsp. *pallasii* (Lehm.) Smith & Forrest	Switzerland, cultivated at Botanical Garden of Zurich, #20050242, raised from seeds obtained from the Botanical Garden Greifswald, Germany, originally from Russia, W Siberia, Altai	no voucher	HM629064	HM628929	HM628999
21	*Primula elatior* subsp. *pseudoelatior* (Kusn.) Smith & Forrest	Georgia, Racha-Lechkhumi Great Caucasus from Shovi along the road to Mamisoni pass, 42°39’N 43°43’E, 2280-2500 m, 13 Jul 2002	Schneeweiss 8009 (WU)	HM629157	HM628933	HM629003
22	*Primula elatior* subsp. *pseudoelatior* (Kusn.) Smith & Forrest	Georgia, Samtshkhe-Javakheti, Tskhatsquaro ugheltekhili ca. 8 km S Bakuriani, 1 km E of pass, 41°42’04’’N 43°31’02’’E, 2100-2500 m, 22 May 2001	Schneeweiss 6680 (WU)	HM629109	HM628932	HM629002
23	*Primula elatior* subsp. *pseudoelatior* (Kusn.) Smith & Forrest	Switzerland, cultivated at Botanical Garden of Zurich, #20071042, raised from seeds obtained from the Botanical Garden Vácrátót, Hungary	no voucher	HM629070	HM628931	HM629001
1	*Primula grandis* Trautv.	Cultivated at Tromsø Botanical Garden (Arktisk-Alpin Botanisk Hage) #92-280, 1995	Photo voucher at Tromsø BG	HM629086	HM628934	HM629004
2	*Primula grandis* Trautv.	Cultivated by Ron McBeath, Lamberton Nusery, Berwickshire, Scotland, 2000	Mast 716 (Z)	HM629088	HM628935	HM629005
1	*Primula juliae* Kusnetsow	Switzerland, cultivated at Botanical Garden of Zurich, # 19780155, 20 Apr 2000	Mast 420 (Z)	HM629108	HM628937	HM629007
2	*Primula juliae* Kusnetsow	Georgia, Lagodechi, Shzoma gorge, 5 May 2000	Davlianidze 1 (Z)	HM629139	HM628938	HM629008
3	*Primula juliae* Kusnetsow	Georgia, Lagodechi, Shzoma gorge, 21 Jun 2000?	Davlianidze 6721/00 (Z)	HM629143	HM628939	HM629009

1	*Primula megaseifolia* Boiss. & Bal.	Cultivated by Mattingley's Cluny House Gardens, Aberfeldy, Perthshire, Scotland, 2000	Mast s.n. (photo voucher Z)	HM629115	HM628940	HM629010
2	*Primula megaseifolia* Boiss. & Bal.	Cultivated by Ian Scott, 2000	Ian Scott s.n. (photo voucher, Z)	HM629145	HM628941	HM629011
3	*Primula megaseifolia* Boiss. & Bal.	Turkey, Artvin-Arhavi, 20 Apr 1980	Calis s.n. (Z)	HM629163	HM628942	HM629012
	Primula renifolia Volg.	Russia, Cherkessk, Tberdinsky State Reserve	F.V. 861 (MW)	HM629172	HM628943	HM629013
1	*Primula veris* L.	Switzerland, cultivated at the Botanical Garden of Zurich, #20070687, originally from Hortus Botanicus Tallinnensis, Estland	no voucher	HM629069	HM628947	HM629018
2	*Primula veris* L.	Switzerland, cultivated at the Botanical Garden of Zurich, #20090291, originally from France, Haute-Savoie, Marignier	no voucher	HM629074	HM628948	HM629019
3	*Primula veris* L.	Switzerland, cultivated at the Botanical Garden of Zurich, #20090351, originally from Poland, Winiary Zagojskie-Steppe, Swietokrzyskie	Schmidt-Lebuhn 1275 (Z)	HM629083	HM628949	HM629020
4	*Primula veris* L.	Switzerland, cultivated at the Botanical Garden of Zurich, #20090362, originally from France, Côte d'Or	Schmidt-Lebuhn 1276 (Z)	HM629084	HM628950	HM629021

CHAPTER VI: CONCLUDING REMARKS

Through the research in this thesis, I advocate an integrative approach to the study of the evolution of plant reproductive diversity. In particular, I have used both phylogenetic and ecological methods to understand the effects of floral traits on plant reproduction, the effects of plant reproduction on the diversification of plant lineages, and on the diversification of floral traits. Several points that were raised during the discussions of the individual chapters can be combined to highlight emerging, overarching questions, and identify major shortcomings in our knowledge of the evolution of plant reproductive diversity.

Evolutionary effects on long and short timescales

In Chapter II, I provide evidence that the evolution of heterostyly promoted long-term diversification of the clade /Primula by decreasing extinction rates, but that the losses of heterostyly over the last million-or-so years actually spurred diversification by promoting speciation rates. Therefore, I suggest that the effect a trait may have on extinction rates might be delayed compared to its effect on speciation rates, and that this can be understood from a population-genetic perspective by focusing on the consequences of decreased effective population sizes associated with shifts to higher inbreeding after the loss of heterostyly. It is a potentially potent explanation to reconcile studies at different time scales that found opposite evolutionary effects of the loss of outcrossing. Moreover, the implied important effect of shifting effective population sizes also helps to explain why we found in Chapter III that floral traits of homostylous species evolve under a more drift-like pattern than flowers of heterostylous species.

These findings and ideas immediately prompt two important avenues for future follow-up studies, to test the hypothesis outlined above. From a population-ecological and -genetic perspective, we could proceed as follows. First, empirical data would need to be gathered to document to what extent effective population sizes actually differ between homostylous and heterostylous species. Secondly, it would need to be understood how much higher the amount of genetic inbreeding is in homostylous species, and how much variation there is among species. In particular, do homostylous species have mixed mating systems, as our ecological study in Chapter IV implies, or are they highly selfing? Thirdly, interpretation of these data need to be corroborated with targeted simulation studies aiming at uncovering to what extent population sizes affect drift, selection, adaptation, and extinction. Finally, it would be important to quantify empirically what the distribution of fitness effects of mutations is, and how shifts in effective population size affects the probability of fixing slightly maladaptive mutations. Are differences between heterostylous and homostylous species large enough to be possibly responsible for the effects implied by our phylogenetic results?

From a phylogenetic perspective, we would need to empirically test the hypothesis that in other systems where the effect of outcrossing mechanisms on diversification has been examined, contrasting results will be obtained when taxonomic sampling is targeted either more or less inclusively. Clearly, the robustness of the results would benefit from a more comprehensive species-level sampling. Additionally, a much better understanding of the behavior of phylogenetic methods is

necessary, on two fronts. First, in chapter II, we demonstrate that the use of different methods for joint tree reconstruction and divergence time estimation affects overall tree shape, that is, the relative length of branches differs at different depths in the tree, with important effects on diversification rate estimation. The signal of extinction in a phylogeny under a constant rate birth-death model entails the observation that, toward the present, an increasingly disproportionate number of lineages has arisen (these include the lineages for which the waiting time until extinction has not passed yet). Therefore, any artifact that affects branch length estimation may well distort the accuracy of estimating diversification dynamics. In that respect, it is somewhat of a relief that we could demonstrate in Chapter V that misspecified branch length priors only affect the scaling of the tree, rather than the pattern of branch lengths within the tree, although the results of Chapter V should nevertheless be alarming when estimation of absolute diversification rates is the objective of a phylogenetic study. The second important unknown property of current phylogenetic methods is their sensitivity to estimating diversification dynamics at different "time-depths". Since extinction is estimated based on a difference in the observed average branch length deeper vs. shallower in the phylogeny, it is important to understand how extinction-rate estimates are affected when the entire phylogeny entails only young lineages. Jointly, these proposed phylogenetic, population genetic and plant-mating investigations seem to have the potential to uncover a putatively central phenomenon in macroevolution, namely that gains and losses of traits during evolution may be generating distinctly different patterns of diversity over short and long time scales (or perhaps over long and very long time scales).

Complex interactions between reproductive traits

Besides points relevant to mating systems and diversification dynamics emergent mainly from Chapters II, III, and V, I would like to highlight an issue related to variation in floral traits and reproductive patterns, emergent from Chapters III and IV, which has important implications for the trajectory of mating system evolution. In Chapter IV, I relate complex patterns of positions of anthers and stigma within flowers and their distance (herkogamy), all of which change during anthesis, to the reproductive success of *Primula halleri* in conditions of open pollination, pollinator exclusion and emasculation. Interestingly, I found that the total number of seeds produced per flower in open-pollinated plants differs among herkogamy classes, such that plants with less herkogamy have a higher reproductive output. In fact, the implied selection coefficient on herkogamy is -0.41, which is comparatively quite strong selection against herkogamy. Given that crossing experiments suggested that fitness differences of selfed versus outcrossed individuals are presumably relatively minor, these results imply that selection should eradicate herkogamy -- yet some degree of herkogamy is common among homostylous species (Chapter III). This discrepancy between theory and data important, because it suggests that more complex selective pressures act on anther and stigma position than just those emergent from seed set, selfing rate and inbreeding depression alone. In particular, it would be very interesting to investigate the fitness effects of individual floral traits much more comprehensively. Besides focusing on the effects of single traits on seed set and maternal fitness, it would be vital to include a focus on pollen export and paternal fitness, for instance following the approach of pollen and ovule fates, or the approach of individual fertility components.

Additionally, a generally overlooked phenomenon in the evolution of floral traits and mating systems is the influence of grouping flowers in inflorescences. *Primula halleri* develops just one or two flowers per day, but each flower may last for more than a week. Therefore, an inflorescence may present flowers of different ages concurrently, and thus, as floral organ positions change during anthesis, may present flowers that have sexual organs positioned at quite different places. Because the position of sexual organs is thought to affect pollen import and export, the rate of flower opening may have implications for the extent of pollinator-mediated, among-flower selfing. This aspect of inbreeding, which was not measured in my experiments, is poorly understood, but is exemplary for a suit of complex interactions between floral traits and developmental rates that ultimately seem to have important fitness effects, and therefore may guide the evolution of floral traits in ways not predicted from the overly simplistic models commonly employed. Jointly, the ideas emergent from my study on *Primula halleri* in Chapter IV suggest that plant mating systems are more complex than can be accurately studied from focusing on selfing rates alone. Congruently, the evolutionary trajectories of homostylous species in general do not seem to fit very well with the idea of a floral design tailored toward high selfing rates, where resource-allocation trade-offs would drive selection for increasingly smaller floral display. Rather, the variability of floral traits that affect mating within species (Chapter IV) and among species (Chapter III) suggest jointly that floral evolution may be more complicated than commonly anticipated.

Finally, throughout my thesis I have often talked about homostylous versus heterostylous species, as if they were two distinct groups, each rather homogeneous. This is also an over simplification, as the results presented in Chapter III clearly demonstrate. In order to resolve whether the variability among homostylous species stems from an increased importance of drift or from multiple distinctly different selective regimes among homostylous species can be resolved by quantifying the selective regimes operating on floral traits of multiple homostylous species more thoroughly. This imlies that field experiments are needed in general, including diligent field observations on plants and pollinators, to address, for instance: How many flowers do pollinators visit within an inflorescence and in what order? How many plants flower concurrently? What is the spatial distribution of early- versus late-flowering individuals within a population? How large is among-flower variance in seed set? How many related species grow nearby and which pollinators fly between them? These are simple questions, and are each relatively straight-forward to address, although they require much time spent in the field. However, such basic biological knowledge can generate hypotheses with implications at micro- and macro-evolutionary levels. When testing these at multiple levels employing both ecological and phylogenetic approaches, an overarching understanding of evolutionary patterns and processes may emerge. And that should be the target.

To conclude this thesis, I would like to stress that ultimately, understanding of the evolution of diversity requires documentation of biological diversity in the natural world around us. As a collegue put it, we should not forget to every now and then *"pitch your tent next to a plant for three weeks and come back with some real knowledge"* – something I wish I did more often.

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Society for the Study of Evolution, Portland, Oregon, July 2010 (oral). "Do homostylous species outcross? Variation of anther-stigma separation and mating in Primula halleri"

Federation of European Biological Systematic Societies, Leiden, Netherlands, August 2009 (poster)

European Society for Evolutionary Biology, Torino, Italy, August 2009 (poster)

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